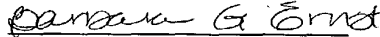


10019258 092302
JC07 Rec'd PCT/PTO 28 DEC 2001

FORM PTO-1390		U.S. Department of Commerce Patent and Trademark Office	Attorney's Docket No. 1181-256
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371			U.S. Application No. (if known, see 37 CFR 1.5) 10/019258
INTERNATIONAL APPLICATION NO. PCT/GB00/02512	INTERNATIONAL FILING DATE June 27, 2000	PRIORITY DATE CLAIMED June 28, 1999	
TITLE OF INVENTION Methods of Cloning and Producing Fragment Chains with Readable Information Content			
APPLICANT(S) FOR DO/EO/US LEXOW, Preben			
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:			
1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371. 2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371. 3. <input checked="" type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1). 4. <input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date. 5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2)) a. <input type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau). b. <input checked="" type="checkbox"/> has been transmitted by the International Bureau. c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US). 6. <input type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)). 7. <input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)) a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau). b. <input type="checkbox"/> have been transmitted by the International Bureau. c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. d. <input checked="" type="checkbox"/> have not been made and will not be made. 8. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)). 9. <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). 10. <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).			
ITEMS 11. TO 16. below concern other document(s) or information included:			
11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98. 12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included. 13. <input checked="" type="checkbox"/> A FIRST preliminary amendment. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment. 14. <input type="checkbox"/> A substitute specification. 15. <input type="checkbox"/> A change of power of attorney and/or address letter. 16. <input checked="" type="checkbox"/> Other items or information: Courtesy copy of International Application No. PCT/GB00/02512 w/ attached International Search Report; Form PCT/IPEA/416 w/4 amended sheets; Form PCT/RO/101; Forms PCT/IPEA/401 and PCT/IPEA/408; Form PCT/ISA/220; Forms PCT/IB/308 and PCT/IB/332; Response to Written Opinion dated October 12, 2001; Request for correction of Request form under Rule 91.1 PCT dated December 6, 2000.			

U.S. APPLICATION NO. (If known, see 37 CFR 1.50) 10/019258		INTERNATIONAL APPLICATION NO. PCT/GB00/02512		ATTORNEY DOCKET NO. 1181-256	
17. <input checked="" type="checkbox"/> The following fees are submitted: Basic National Fee (37 CFR 1.492)(a)(1)-(5): Search Report has been prepared by the EPO or JPO \$ 890.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) \$ 710.00 No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) \$ 740.00 Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$ 1,040.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) \$ 100.00				<u>CALCULATIONS</u>	<u>PTO USE ONLY</u>
ENTER APPROPRIATE BASIC FEE AMOUNT =				\$ 890.00	
Surcharge of \$130.00 for furnishing the oath or declaration later than [] 20 [] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				\$	
Claims	Number Filed	Number Extra	Rate		
Total Claims	28 - 20 =	8	X \$18.00	\$ 144	
Independent Claims	3 - 3 =	0	X \$84.00	\$	
Multiple dependent claim(s) (if applicable)			+ \$280.00	\$280.00	
TOTAL OF ABOVE CALCULATIONS =				\$1,278.00	
Reduction by 1/2 for filing by small entity, if applicable. Applicant claims small entity status. (Note 37 CFR 1.9, 1.27, 1.28).				\$639.00	
SUBTOTAL =				\$639.00	
Processing fee of \$130.00 for furnishing the English translation later [] 20 [] 30 than months from the earliest claimed priority date (37 CFR 1.492(f)).				+	\$
TOTAL NATIONAL FEE =				\$639.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property				+	\$
TOTAL FEES ENCLOSED =				\$639.00	
				Amount to be refunded	\$
				charged	\$
a. <input checked="" type="checkbox"/> A check in the amount of \$ <u>639.00</u> to cover the above fees is enclosed. b. <input type="checkbox"/> Please charge my Deposit Account No. 02-2135 in the amount of \$ _____ to cover the above fees. A duplicate copy of this sheet is enclosed. c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 02-2135. A duplicate copy of this sheet is enclosed. d. <input type="checkbox"/> Payment by credit card. (Form PTO-2038 enclosed.) NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.					
SEND ALL CORRESPONDENCE TO: Barbara G. Ernst Rothwell, Figg, Ernst & Manbeck 555 13th St., N.W. Washington, D.C. 20004 Phone: 202/783-6040			<div style="text-align: right;">  Signature <hr/> Barbara G. Ernst Name <hr/> 30,377 Registration Number </div>		

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE	Application Number	PCT/GB00/02512
	Filing Date	June 27, 2000
	First Named Inventor	Preben LEXOW
	Group Art Unit	Unassigned
	Examiner Name	Unassigned
	Attorney Docket Number	1181-256
Title: METHODS OF CLONING AND PRODUCING FRAGMENT CHAINS WITH READABLE INFORMATION CONTENT		

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
 Washington, D.C. 20231

Dear Sir:

Please enter the following amendments before calculation of the filing fee and examination the merits.

IN THE CLAIMS:

Please amend claims 8-13 as follows:

8. (Amended) A method as claimed in claim 1, 2 or 3 wherein said fragments are each between 8 and 25 bases in length.

9. (Amended) A method as claimed in claim 1, 2 or 3 wherein n is at least 10.

10. (Amended) A method of synthesizing a double stranded nucleic acid molecule comprising at least the steps of:

- 1) generating fragment chains according to the method defined in claim 1, 2 or 3;

- 2) optionally generating single stranded regions at the end of said fragment chains, wherein said single stranded regions are complementary to the single stranded regions on said fragment chains thus forming complementary pairs of single stranded regions;
- 3) contacting said fragment chains with one another, simultaneously or consecutively, to effect binding of said complementary pairs of single stranded regions.

11. (Amended) A nucleic acid molecule produced according to a method as defined in claim 1, 2 or 3, or a single stranded nucleic acid molecule thereof.

12. (Amended) A method of identifying the code elements contained in a nucleic acid molecule prepared according to a method as defined in claim 1, 2 or 3, wherein a probe, carrying a signaling means, specific to one or more code elements, is bound to said nucleic acid molecule and a signal generated by said signalling means is detected, whereby said one or more code elements may be identified.

13. (Amended) A library of fragments as defined in claim 1, 2 or 3, comprising $(n)_m$ fragments, wherein n is as defined in claim 1, 2 or 3 and corresponds to the length of chain that said library may produce, and m is an integer corresponding to the number of possible code elements or combinations thereof, such that fragments corresponding to all possible code elements for each position in the final chain are provided.

IN THE ABSTRACT

Please add the following abstract on the accompanying separate sheet.

The accompanying amendments are being made to eliminate multiple dependencies in the claims, and place the Abstract in better U.S. form.

RESPECTFULLY SUBMITTED,					
NAME AND REG. NUMBER	Barbara G. Ernst, Registration No. 30,377				
SIGNATURE	Barbara G. Ernst			DATE	Dec. 28, 2001
Address	Rothwell, Figg, Ernst & Manbeck Suite 701-East, 555 13th Street, NW				
City	Washington	State	D.C.	Zip Code	20004
Country	U.S.A.	Telephone	202-783-6040	Fax	202-783-6031

Attachments: Version of amended claims to show changes made and Abstract

Amended Claims: Version to show changes made

8. (Amended) A method as claimed in [any one of claims 1 to 7] claim 1, 2 or 3 wherein said fragments are each between 8 and 25 bases in length.

9. (Amended) A method as claimed in [any one of claims 1 to 8] claim 1, 2 or 3 wherein n is at least 10.

10. (Amended) A method of synthesizing a double stranded nucleic acid molecule comprising at least the steps of:

- 1) generating fragment chains according to the method defined in [any one of claims 1 to 9] claim 1, 2 or 3;
- 2) optionally generating single stranded regions at the end of said fragment chains, wherein said single stranded regions are complementary to the single stranded regions on said fragment chains thus forming complementary pairs of single stranded regions;
- 3) contacting said fragment chains with one another, simultaneously or consecutively, to effect binding of said complementary pairs of single stranded regions.

11. (Amended) A nucleic acid molecule produced according to a method as defined in [any one of claims 1 to 10] claim 1, 2 or 3, or a single stranded nucleic acid molecule thereof.

12. (Amended) A method of identifying the code elements contained in a nucleic acid molecule prepared according to a method as defined in [any one of claims 1 to 10] claim 1, 2 or 3, wherein a probe, carrying a signalling means, specific to one or more code elements, is bound to said nucleic acid molecule and a signal generated by said signalling means is detected, whereby said one or more code elements may be identified.

13. (Amended) A library of fragments as defined in [any one of claims 1 to 12] claim 1, 2 or 3, comprising $(n)_m$ fragments, wherein n is as defined in [any one of claims 1 to 12] claim 1, 2 or 3 and corresponds to the length of chain that said library may produce, and m is an integer

corresponding to the number of possible code elements or combinations thereof, such that fragments corresponding to all possible code elements for each position in the final chain are provided.

The present invention provides a method of attaching a fragment of a first nucleic acid molecule to a second nucleic acid molecule using adapters to mediate the binding particularly in methods of cloning, methods of producing fragment chains with a readily readable information content, particularly comprising fragments corresponding to code, such as alphanumeric code, the nucleic acid molecules thus produced and kits for performing such methods.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE	Application Number	US 10/019,258
	Filing Date	Dec. 28, 2001
	First Named Inventor	LEXOW, Preben
	Group Art Unit	Unassigned
	Examiner Name	Unassigned
	Attorney Docket Number	1181-256
Title: METHODS OF CLONING AND PRODUCING FRAGMENT CHAINS WITH READABLE INFORMATION CONTENT		

**SECOND PRELIMINARY AMENDMENT and
RESPONSE TO NOTIFICATION OF MISSING REQUIREMENTS
UNDER 35 U.S.C. §371**

Assistant Commissioner for Patents
Box PCT
Washington, DC 20231

Dear Sir:

In response to the Notification of Missing Requirements dated March 22, 2002 (copy enclosed) enclosed is the Declaration and Power of Attorney and a check for \$785.00 to cover the \$65.00 surcharge for late filing of the declaration and the \$720.00 four-month extension of time fee. Please charge any additional fees to deposit account number 02-2135 in the name of Rothwell, Figg, Ernst & Manbeck.

Attached is the sequence listing in paper and computer readable form.

Entry of the following amendments is respectfully requested.
IN THE SPECIFICATION:

Please enter the attached Sequence Listing submitted herewith.

Amend the specification as shown on the following pages.

09/25/2002 MKAYPAGH 00000039 10019258

01 FC:254

65.00 OP

09/25/2002 MKAYPAGH 00000039 10019258

02 FC:218

720.00 OP

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Marked-up copies of the original text of the amended specification are attached to this amendment. Material inserted is indicated by underline (underline) and material deleted is indicated by angled brackets (<angled brackets>).

Clean copy of the amended specification (paragraph on page 14 at line 26-page 15 at line 11)

To increase the number of permutations in an adapter library, two separate oligonucleotide libraries may be generated, one with single stranded oligonucleotides with regions that will correspond to the single stranded region of the first nucleic acid molecule fragment and the second library with single stranded oligonucleotides with regions that will correspond to the single stranded region of the second nucleic acid molecule (e.g. vector). However in common in each member of the library is a complementary region, such that when one member from the first library is selected and combined with a member of the second library, they will hybridize leaving free the relevant single stranded regions. Thus for example to generate an adapter with an AA overhang and a TC overhang to bind to the first and second nucleic acid molecules respectively, members of the different libraries such as GGCCCCCNAA[SEQ ID NO:1] may be combined with 3'-TCNNNCCGGGG-5'[SEQ ID NO:2] to form:

GGCCCCCNAA[SEQ ID NO:1]

TCNNNCCGGGG[SEQ ID NO:2]

which exhibits the appropriate overhangs. When using only two 16 member libraries this allows the production of 256 different adapters.

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Clean copy of the amended specification (paragraph on page 16 at line 20-page 17 at line 11)

Over 100 classes of IIS restriction endonucleases have been identified and there are large variations both with respect to substrate specificity and cleaving pattern. In addition, these enzymes have proved to be well suited to "module swapping" experiments so that one can create new enzymes for particular requirements (Huang-B, et al.; J-Protein-Chem. 1996, 15(5):481-9, Bickle, T.A.; 1993 in Nucleases (2nd edn), Kim-YG et al.; PNAS 1994, 91:883-887). In these experiments the binding domain of transcription factor *Sp1* was merged with the cleavage domain of *FokI* to construct a class IIS restriction endonuclease that makes a 4-base overhang with *Sp1* sites. In other experiments a class IIS restriction endonuclease that cuts outside the binding sites of transcription factor Ultrabithorax was generated. Corresponding experiments have been conducted on class I enzymes. By merging the N-terminal part of the *hsdS* sub-unit of *StyR* 1241 (which recognizes GAAN₆RTTC[SEQ ID NO:82]) with the C-terminal part of the *hsdS* sub-unit of *StyR* 1241 (which recognizes TCAN₇RTTC[SEQ ID NO:83]) a new enzyme that recognizes the sequence GAAN₆RTTC[SEQ ID NO:84] was constructed. Several other experiments have been carried out with similar success. Unlike in the case of ordinary class II enzymes, it is therefore reasonable to assume that a number of new IIS and IP restriction enzymes can be constructed and adapted to cloning requirements that may arise in the future. Very many combinations and variants of these enzymes can therefore be used according to the principles described herein.

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Clean copy of the amended specification (paragraph on page 44 at line 30-page 45 at line 25)

The following examples are given by way of illustration only in which the Figures referred to are as follows:

Figure 1 shows a schematic representation of how the method of the invention may be used to introduce an insert into a vector, in which the insert is cleaved from the first nucleic acid molecule, associated with adapters and ligated thereto and then ligated into the vector;

Figure 2 shows the production of a fragment chain using 8 "O" and "I" starting fragments with different overhangs (aaaaaaaaa[SEQ ID NO:100], aaaaaaaaaac[SEQ ID NO:54], aaaaaaacg[SEQ ID NO:57], ccccccccccg[SEQ ID NO:59], cccccccccgcg[SEQ ID NO:56], ccccccccttt[SEQ ID NO:53], ggggggggaaa[SEQ ID NO:51], ggggggggaac[SEQ ID NO:52], ggggggggccg[SEQ ID NO:55], ttttttttcg[SEQ ID NO:60], ttttttttgcg[SEQ ID NO:58], tttttttttt[SEQ ID NO:101]);

Figure 3 shows the production of a 64 fragment chain in which 8 chains are produced comprising 8 fragments each, in which the termini of chains 1 and 2, and 2 and 3 etc. are complementary such that they may be ligated together (aaaaaaaaa[SEQ ID NO:100], aaaaaaaaaaaaaa[SEQ ID NO:102], aaaggggggggaaa[SEQ ID NO:61], aacaaaaaaaaaaaaa[SEQ ID NO:62], aacgggggggggaaa[SEQ ID NO:103], cttccccccccccg[SEQ ID NO:104], ctttttttttttcg[SEQ ID NO:105], ggggggggaaa[SEQ ID NO:51], gttccccccccccg[SEQ ID NO:65], gtttttttttttcg[SEQ ID NO:66], tttccccccccccg[SEQ ID NO:63], ttttttttttttcg[SEQ ID NO:64]);

Figure 4 shows 3 techniques for mixing "O", "I" fragments from a library of fragments ordered for each position, in which in A)

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appropriate fragments are selected by aspiration from appropriate wells, B) appropriate fragments are released from the library is wells and C) a flow cytometer is used to direct appropriate droplets to the mixing chamber;

Figure 5 shows PCR amplification of signal chain 1-0-1-0-0 using SP6 and T7 primers. Lane 1: 1 µg of 1 kb DNA ladder (Gibco BRL), Lane 2: 10 µl of PCR amplified fragment chain DNA using SP6 and T7 primers. Lane 3: Same as lane 2 except for the use of SP6 and T7-Cy5 primers; and

Figure 6 shows the use of primer pairs during the process of amplification to join together fragment chains.

Clean copy of the amended specification (paragraph on page 48 at lines 21-34)

Materials:

Oligonucleotides used to address *PhiX174* overhangs:

BbvI overhang 1a:

5'- CGA GCG CCT CCA GTG CAG CGG AG[SEQ ID NO:3]

BbvI overhang 5a:

5'- TATC GCG CCT CCA GTG CAG CGG AG[SEQ ID NO:4]

BbvI overhang 6b:

5'- CTCT GCG CCT CCA GTG CAG CGG AG[SEQ ID NO:5]

BbvI overhang 6(delC):

5'- CTCT CTC CGC TGC ACT GGA GGC GC[SEQ ID NO:6]

BbvI overhang 7a:

5'- CAAC GCG CCT CCA GTG CAG CGG AG[SEQ ID NO:7]

BbvI overhang 9b:

5'- GGTA GCG CCT CCA GTG CAG CGG AG[SEQ ID NO:8]

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Clean copy of the amended specification (paragraph on page 49 at lines 1-5)

Oligonucleotides used to address pUC19 overhangs:

Cloning site 1a

5'- AAGAG CTC CGC TGC ACT GGA GGC GC[SEQ ID NO:9]

Cloning site 1b

5'- CTCTT CTC CGC TGC ACT GGA GGC GC[SEQ ID NO:10]

Clean copy of the amended specification (paragraph on page 53 at line 11-page 54 at line 6)

In this Example, the location of the binding motifs of the initiation linkers is shown below:

<i>FokI</i>	-----GGATG----
<i>Bst71I</i>	--GCAGC-----
<i>HgaI</i>	-----GACGC
<i>BplI</i>	-----GAG-----CTC-----
<i>BaeI</i>	-----CYATG----CA-----
<i>CjeI</i>	-----CCA-----GT-----
<i>HaeIV</i>	-----GAY-----RTC-----
Consensus	--GCAGCGACCATGAGTCCA-CTC--GTGGATGACGC[SEQ ID NO:11]

Initiation linkers:

X=0: 5' --GCAGCGACCATGAGTCCA-CTC--GTGGATGPPPPPP[SEQ ID NO:12]
3' --CGTCGCTGGTACTCAGGT-GAG--CACCTAC[SEQ ID NO:69]
X=1: 5' --GCAGCGACCATGAGTCCA-CTC--GTGGATG-PPPPPP[SEQ ID NO:13]
3' --CGTCGCTGGTACTCAGGT-GAG--CACCTAC-[SEQ ID NO:70]
X=2: 5' --GCAGCGACCATGAGTCCA-CTC--GTGGATG--PPPPPP[SEQ ID NO:14]

3' --CGTCGCTGGTACTCAGGT-GAG--CACCTAC--[SEQ ID NO:71]
X=3: 5' --GCAGCGACCATGAGTCCA-CTC--GTGGATG---PPPPPP[SEQ ID NO:15]
3' --CGTCGCTGGTACTCAGGT-GAG--CACCTAC---[SEQ ID NO:72]
X=4: 5' --GCAGCGACCATGAGTCCA-CTC--GTGGATGACGCPPPPPP[SEQ ID NO:16]
3' --CGTCGCTGGTACTCAGGT-GAG--CACCTACTGCG[SEQ ID NO:73]
X=5: 5' --GCAGCGACCATGAGTCCA-CTC--GTGGATGACGC-PPPPPP[SEQ ID
NO:17]
3' --CGTCGCTGGTACTCAGGT-GAG--CACCTACTGCG-[SEQ ID NO:74]
X=6: 5' --GCAGCGACCATGAGTCCA-CTC--GTGGATGACGC--PPPPPP[SEQ ID
NO:18]
3' --CGTCGCTGGTACTCAGGT-GAG--CACCTACTGCG--[SEQ ID NO:75]
X=7: 5' --GCAGCGACCATGAGTCCA-CTC--GTGGATGACGC---PPPPPP[SEQ ID
NO:19]
3' --CGTCGCTGGTACTCAGGT-GAG--CACCTACTGCG---[SEQ ID NO:76]
X=8: 5' --GCAGCGACCATGAGTCCA-CTC--GTGGATGACGC----PPPPPP[SEQ ID
NO:20]
3' --CGTCGCTGGTACTCAGGT-GAG--CACCTACTGCG----[SEQ ID NO:77]
X=9: 5' --GCAGCGACCATGAGTCCA-CTC--GTGGATGACGC-----PPPPPP[SEQ ID
NO:21]
3' --CGTCGCTGGTACTCAGGT-GAG--CACCTACTGCG-----[SEQ ID NO:78]

Clean copy of the amended specification (paragraph on page 54 at lines 21-35)

```

FokI:      5'-----GGATG
            3'-----CCTACNNNN
Bst71I:    5'-----GCAGC
            3'-----CGTCGNNNN
HgaI:      5'-----GACGC
            3'-----CTGCGNNNN[SEQ ID NO:79]

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SplI: 5'-----GAG-----CTCNNNNN
3'-----CTC-----GAG
BaeI: 5'-----CCATG-----CANNNNN
3'-----GGTAC-----GT
HaeIV: 5'-----GAC-----GTCNNNNNN
3'-----CTG-----CTG
CjeI: 5'-----CCA-----GTNNNNNN
3'-----GGT-----CA

Clean copy of the amended specification (paragraph on page 55 at lines 28-36)

The 3'-GAGTGC overhang is then ligated with the X=3 initiation linker and the GTGAA-3' overhang is ligated with the CACTT-3' overhang on the target DNA molecule:

5'--GCAGCGACCATGAGTCCA-CTC--GTGGATG---PPPPPP[SEQ ID NO:15]
3'--CGTCGCTGGTACTCAGGT-GAG--CACCTAC---QQQQQQ[SEQ ID NO:85]
-----GTGAA-----3'
-----CACTT-----5'

Clean copy of the amended specification (paragraphs on page 56 at line 15-page 58 at line 7)

Method 1

Two IIS enzymes that generate 5'-4 base overhangs (*BbsI* and *Esp3I*):

5'..VVVVVVVVGAGC-GAGACG-----GAAGAC--GAGCIIIIIIIIII 3'[SEQ ID NO:86]

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3' VVVVVVVVCTCG-CTCTGC-----CTTCTG--CTCGIIIIIIIIII..5' [SEQ ID NO:87]

After cleavage with *Bbs*I and *Esp*3I:

..VVVVVVVV + GAGC-GAGACG-----GAAGAC-- [SEQ ID NO:88] +
VVVVVVVVCTCG -CTCTGC-----CTTCTG--CTCG [SEQ ID NO:89]

GAGCIIIIIIIIII
IIIIIIIIII..

After ligation with T4 DNA ligase:

GAGC-GAGACG-----GAAGAC-- [SEQ ID NO:88] +
-CTCTGC-----CTTCTG--CTCG [SEQ ID NO:89]

..VVVVVVVVGAGCIIIIIIIIII [SEQ ID NO:90]
VVVVVVVCTCGIIIIIIIIII.. [SEQ ID NO:91]

Method 2

One IIS enzyme that generates two 3' 3 base overhangs (*Bsa*XI):

5'..VVVVVVVVGAG-----AC-----CTCC-----GAGIIIIIIIIII 3' [SEQ ID NO:92]
3' VVVVVVVVCTC-----TG-----GAGG-----CTCIIIIIIIIII..5' [SEQ ID NO:93]

After cleavage with *Bsa*XI:

..VVVVVVVVGAG + -----AC-----CTCC-----GAG [SEQ ID NO:94]
VVVVVVVV CTC-----TG-----GAGG----- [SEQ ID NO:95]

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+ IIIIIIIIII
 CTCIIIIIIIIII..

After ligation with T4 DNA ligase:

-----AC-----CTCC-----GAG[SEQ ID NO:94] +
CTC-----TG-----GAGG-----[SEQ ID NO:95]

..VVVVVVVVGAGIIIIIIIIII
VVVVVVVVCTCIIIIIIIIII..

Method 3

One IIS enzyme that generates blunt ends (*MlyI*):

5'..VVVVVVVV-----GAGTC-----IIIIIIIIII 3'[SEQ ID
NO:96]
3' VVVVVVVV-----CTGAG-----IIIIIIIIII..5'[SEQ ID
NO:96]

After cleavage with *MlyI*:

..VVVVVVVV + -----GAGTC-----[SEQ ID NO:97] +
VVVVVVVV -----CTGAG-----[SEQ ID NO:97]

IIIIIIIIII
IIIIIIIIII..

After ligation with T4 DNA ligase:

-----GAGTC-----[SEQ ID NO:97] +
-----CTGAG-----[SEQ ID NO:97]

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..VVVVVVVVVIIIIIIIIIII
VVVVVVVVVIIIIIIIIIII..

Clean copy of the amended specification (paragraph on page 71 at line 14-page 72 at line 4)

Based upon the overhang pairs, a set of five library components was made by annealing complementary oligonucleotides in separate tubes:

signal 1:

5'-TAATACGACTCACTATAACCACAAGTTTGTACAAAAAAGCAGGCTCTATTC-3' [SEQ ID NO:22]

and

5'-TAGGAAGAATAGAGCCTGCTTTTTTGTACAAACTTGTGGTATAGTGAGTCGTATTA-3' [SEQ ID NO:23];

signal 2:

5'-TTCCTATGCAGTGGACCACTTTGTACAAGAAAGCTGGGTTGCAGT-3' [SEQ ID NO:24]

and 5'-GCAACTACTGCAACCCAGCTTTCTTGTACAAAGTGGTCCCACTGCA-3' [SEQ ID NO:25];

signal 3:

5'-AGTTGCTTGACGCCACAAGTTTGTACAAAAAAGCAGGCTTTGACG-3' [SEQ ID NO:26]

and 5'-CGACATCGTCAAAGCCTGCTTTTTTGTACAAACTTGTGGCGTCAA-3' [SEQ ID NO:27];

signal 4:

5'-ATGTCGAAGGGCGGACCACTTTGTACAAGAAAGCTGGGTAAGGGC-3' [SEQ ID NO:28]

and 5'-GACAGGGCCCTTACCCAGCTTTCTTGTACAAAGTGGTCCGCCCTT-3' [SEQ ID NO:29];

signal 5:

5'-CCTGTCATGTGGACCACTTTGTACAAGAAAGCTGGGTTTCTATAGTGTCACCTAAATC-3' [SEQ ID NO:30] and

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5'-GATTTAGGTGACACTATAGAAACCCAGCTTCTTGACAAAGTGGTCCACAT-3' [SEQ ID NO:31];

T7: 5'-TAATACGACTCACTATACCA-3' [SEQ ID NO:32];

T7-CyS primer: 5'-TAATACGACTCACTATA-3' [SEQ ID NO:33]; and

SP6 primer: 3'-AAGATATCACAGTGGATTTAG-5' [SEQ ID NO:34].

The library components (4 pmol each) were then mixed together and ligated using 100 U T4 DNA ligase (NEB) in 1X ligase buffer at 25 °C for 15 minutes. The ligase was then inactivated at 65 °C for 20 min.

Clean copy of the amended specification (paragraph on page 73 at lines 10-26)

Materials:

Oligonucleotides are selected which bind to the fragment chain and also serve as primers. Thus for example, for adjacent chains may be bound using for example the following primer pairs:

fragment chain 2 terminal (*with bound primer*):

5'TTCTATAGTGTCACCTAAATC3' [SEQ ID NO:35]

3'AAGATATCACAGTGGATTTAGCCTACCAGTACATCCAACGGCAACT5' [SEQ ID NO:36]

fragment chain 3 terminal (*with bound primer*):

5'GTCATGTAGGTTGCCGTTGATCCATCCTAATACGACTCACTATAGCA3' [SEQ ID NO:37]

3'ATTATGCTGAGTGATATCGT5' [SEQ ID NO:38]

The above exemplified primer regions are complementary and may thus be bound together.

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Clean copy of the amended specification (paragraph on page 75 at lines 12-18)

Gene A has the following sequence at its first and last five bases (marked by underlining).

5'...GCTGGAGGCCTCCACTATGAAATCGCGTAGAG...[SEQ ID NO:80]
3'...CGACCTCCGGAGGTGATACTTTAGCGCATC.....[SEQ ID NO:98]
.....CTGGCGGAAATGAGAAAATTCGACCTA...3'[SEQ ID NO:81]
...ACGACCGCCTTTTACTCTTTTAAGCTGG.....5'[SEQ ID NO:99]

Clean copy of the amended specification (paragraph on page 76 at line 1-page 77 at line 2)

Materials:

Initiation linker 1 (s):

5'ATT CGG TCG AGA TGC TCT CA3'[SEQ ID NO:39]

Initiator linker 1 (as):

5'CGA CTG AGA GCA TCT CGA CCG AAT3'[SEQ ID NO:40]

Initiation linker 2 (s):

5'GCG TTA CTG AGC GTA GCT CTG3'[SEQ ID NO:41]

Initiator linker 2 (as):

5'CTC TCA GAG CTA CGC TCA GTA ACG C3'[SEQ ID NO:42]

Propagation linker (s):

5'TGC TGC AGG AGC GAA TCT CNN NNN3'[SEQ ID NO:43]

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Propagation linker (as):

5'GAG ATT CGC TCC TGC AGC A3'[SEQ ID NO:44]

Labeling linker 2 (s):

5'CTC TTG CTA TAG TGA GTC GTA TTA3'[SEQ ID NO:45]

Labeling linker 2 (as):

5'TAA TAC GAC TCA CTA TAG CA3'[SEQ ID NO:46]

Termination linker 1 (s):

5'AAG AGC TCA GGT CAT TGA CGT AGC TAT GAA3'[SEQ ID NO:47]

Termination linker 1/2 (as):

5'AGC TAC GTC AAT GAC CTG AG3'[SEQ ID NO:48]

Termination linker I (short version):

5'AAG AGA TGA A3'[SEQ ID NO:49]

Termination linker 2 (s):

5'ACC GCT CAG GTC ATT GAC GTA GCT TCA TT3'[SEQ ID NO:50]

Termination linker 2 (short version):

5'ACC GTC ATT3'

REMARKS

In response to a Notification of Missing Requirements under 35 U.S.C. §371 dated March 22, 2002 (a response copy is attached), an initial Sequence Listing is submitted, and its entry into the application is respectfully requested. Pursuant to 37 CFR § 1.821(e), an initial computer-readable form of the Sequence Listing is also submitted, and it is hereby certified

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that the contents of the paper and computer-readable copies of the Sequence Listing are identical and contain no new matter.

The specification has been amended to properly include the sequence identifiers, and correct obvious typographical errors.

RESPECTFULLY SUBMITTED,					
Name and Reg. Number	Barbara G. Ernst Registration No. 30,377				
Signature	Barbara G Ernst		DATE	Sept. 23, 2002	
Address	Rothwell, Figg, Ernst & Manbeck 1425 K Street, N.W., Suite 800				
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Country	U.S.A.	Telephone	202-783-6040	Fax	202-783-6031

1181-256.not_missing_req

Attachments: Marked-Up Copies of Amendments

Paper Copy of Sequence Listing

Copy of PTO Notification of Missing Requirements

Computer Readable Form (diskette) of Sequence

Listing

Over 100 classes of IIS restriction endonucleases have been identified and there are large variations both with respect to substrate specificity and cleaving pattern. In addition, these

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enzymes have proved to be well suited to "module swapping" experiments so that one can create new enzymes for particular requirements (Huang-B, et al.; J-Protein-Chem. 1996, 15(5):481-9, Bickle, T.A.; 1993 in Nucleases (2nd edn), Kim-YG et al.; PNAS 1994, 91:883-887). In these experiments the binding domain of transcription factor *Sp1* was merged with the cleavage domain of *FokI* to construct a class IIS restriction endonuclease that makes a 4-base overhang with *Sp1* sites. In other experiments a class IIS restriction endonuclease that cuts outside the binding sites of transcription factor Ultrabithorax was generated. Corresponding experiments have been conducted on class I enzymes. By merging the N-terminal part of the *hsdS* sub-unit of *StyR* 1241 (which recognizes GAAN₆RTCG[SEQ ID NO:82]) with the C-terminal part of the *hsdS* sub-unit of *StyR* 1241 (which recognizes TCAN₇RTTC[SEQ ID NO:83]) a new enzyme that recognizes the sequence GAAN₆RTTC[SEQ ID NO:84] was constructed. Several other experiments have been carried out with similar success. Unlike in the case of ordinary class II enzymes, it is therefore reasonable to assume that a number of new IIS and IP restriction enzymes can be constructed and adapted to cloning requirements that may arise in the future. Very many combinations and variants of these enzymes can therefore be used according to the principles described herein.

Marked-up copy of the amended specification (paragraph on page 44 at line 30-page 45 at line 25)

The following examples are given by way of illustration only in which the Figures referred to are as follows:

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Figure 1 shows a schematic representation of how the method of the invention may be used to introduce an insert into a vector, in which the insert is cleaved from the first nucleic acid molecule, associated with adapters and ligated thereto and then ligated into the vector;

Figure 2 shows the production of a fragment chain using 8 "O" and "I" starting fragments with different overhangs (aaaaaaaaa[SEQ ID NO:100], aaaaaaaaaac[SEQ ID NO:54], aaaaaaacccg[SEQ ID NO:57], ccccccccccg[SEQ ID NO:59], cccccccccgcg[SEQ ID NO:56], ccccccccttt[SEQ ID NO:53], ggggggggaaa[SEQ ID NO:51], ggggggggaac[SEQ ID NO:52], ggggggggccg[SEQ ID NO:55], ttttttttcg[SEQ ID NO:60], ttttttttgcg[SEQ ID NO:58], tttttttttt[SEQ ID NO:101]);

Figure 3 shows the production of a 64 fragment chain in which 8 chains are produced comprising 8 fragments each, in which the termini of chains 1 and 2, and 2 and 3 etc. are complementary such that they may be ligated together (aaaaaaaaa[SEQ ID NO:100], aaaaaaaaaaaaaa[SEQ ID NO:102], aaaggggggggaaa[SEQ ID NO:61], aacaaaaaaaaaaaaa[SEQ ID NO:62], aacgggggggggaaa[SEQ ID NO:103], cttccccccccccg[SEQ ID NO:104], ctttttttttttcg[SEQ ID NO:105], ggggggggaaa[SEQ ID NO:51], gttccccccccccg[SEQ ID NO:65], gtttttttttttcg[SEQ ID NO:66], tttccccccccccg[SEQ ID NO:63], ttttttttttttcg[SEQ ID NO:64]);

Figure 4 shows 3 techniques for mixing "O", "I" fragments from a library of fragments ordered for each position, in which in A) appropriate fragments are selected by aspiration from appropriate wells, B) appropriate fragments are released from the library is wells and C) a flow cytometer is used to direct appropriate droplets to the mixing chamber;

Figure 5 shows PCR amplification of signal chain 1-0-1-0-0 using SP6 and T7 primers. Lane 1: 1 µg of 1 kb DNA ladder (Gibco BRL),

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Lane 2: 10 µl of PCR amplified fragment chain DNA using SP6 and T7 primers. Lane 3: Same as lane 2 except for the use of SP6 and T7-Cy5 primers; and
Figure 6 shows the use of primer pairs during the process of amplification to join together fragment chains.

Marked-up copy of the amended specification (paragraph on page 48 at lines 21-34)

Materials:

Oligonucleotides used to address *PhiX174* overhangs:

BbvI overhang 1a:

5'- CGA GCG CCT CCA GTG CAG CGG AG[SEQ ID NO:3]

BbvI overhang 5a:

5'- TATC GCG CCT CCA GTG CAG CGG AG[SEQ ID NO:4]

BbvI overhang 6b:

5'- CTCT GCG CCT CCA GTG CAG CGG AG[SEQ ID NO:5]

BbvI overhang 6(delC):

5'- CTCT CTC CGC TGC ACT GGA GGC GC[SEQ ID NO:6]

BbvI overhang 7a:

5'- CAAC GCG CCT CCA GTG CAG CGG AG[SEQ ID NO:7]

BbvI overhang 9b:

5'- GGTA GCG CCT CCA GTG CAG CGG AG[SEQ ID NO:8]

Marked-up copy of the amended specification (paragraph on page 49 at lines 1-5)

Oligonucleotides used to address pUC19 overhangs:

Cloning site 1a

5'- AAGAG CTC CGC TGC ACT GGA GGC GC[SEQ ID NO:9]

Cloning site 1b

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5'- CTCTT CTC CGC TGC ACT GGA GGC GC[SEQ ID NO:10]

**Marked-up copy of the amended specification (paragraph on page 53
at line 11-page 54 at line 6)**

In this Example, the location of the binding motifs of the
initiation linkers is shown below:

<i>FokI</i>	-----GGATG----
<i>Bst71I</i>	--GCAGC-----
<i>HgaI</i>	-----GACGC
<i>BplI</i>	-----GAG-----CTC-----
<i>BaeI</i>	-----CYATG----CA-----
<i>CjeI</i>	-----CCA-----GT-----
<i>HaeIV</i>	-----GAY-----RTC-----
Consensus	--GCAGCGACCATGAGTCCA-CTC--GTGGATGACGC[SEQ ID NO:11]

Initiation linkers:

X=0:	5' --GCAGCGACCATGAGTCCA-CTC--GTGGATGPPPPPP[SEQ ID NO:12]
	3' --CGTCGCTGGTACTCAGGT-GAG--CACCTAC[SEQ ID NO:69]
X=1:	5' --GCAGCGACCATGAGTCCA-CTC--GTGGATG-PPPPPP[SEQ ID NO:13]
	3' --CGTCGCTGGTACTCAGGT-GAG--CACCTAC-[SEQ ID NO:70]
X=2:	5' --GCAGCGACCATGAGTCCA-CTC--GTGGATG--PPPPPP[SEQ ID NO:14]
	3' --CGTCGCTGGTACTCAGGT-GAG--CACCTAC--[SEQ ID NO:71]
X=3:	5' --GCAGCGACCATGAGTCCA-CTC--GTGGATG---PPPPPP[SEQ ID NO:15]
	3' --CGTCGCTGGTACTCAGGT-GAG--CACCTAC---[SEQ ID NO:72]
X=4:	5' --GCAGCGACCATGAGTCCA-CTC--GTGGATGACGCPPPPPP[SEQ ID NO:16]
	3' --CGTCGCTGGTACTCAGGT-GAG--CACCTACTGCG[SEQ ID NO:73]
X=5:	5' --GCAGCGACCATGAGTCCA-CTC--GTGGATGACGC-PPPPPP[SEQ ID NO:17]
	3' --CGTCGCTGGTACTCAGGT-GAG--CACCTACTGCG-[SEQ ID NO:74]

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X=6: 5'--GCAGCGACCATGAGTCCA-CTC--GTGGATGACGC--PPPPPP[SEQ ID
NO:18]
3' --CGTCGCTGGTACTCAGGT-GAG--CACCTACTGCG--[SEQ ID NO:75]
X=7: 5' --GCAGCGACCATGAGTCCA-CTC--GTGGATGACGC---PPPPPP[SEQ ID
NO:19]
3' --CGTCGCTGGTACTCAGGT-GAG--CACCTACTGCG---[SEQ ID NO:76]
X=8: 5' --GCAGCGACCATGAGTCCA-CTC--GTGGATGACGC----PPPPPP[SEQ ID
NO:20]
3' --CGTCGCTGGTACTCAGGT-GAG--CACCTACTGCG----[SEQ ID NO:77]
X=9: 5' --GCAGCGACCATGAGTCCA-CTC--GTGGATGACGC-----PPPPPP[SEQ ID
NO:21]
3' --CGTCGCTGGTACTCAGGT-GAG--CACCTACTGCG-----[SEQ ID NO:78]

**Marked-up copy of the amended specification (paragraph on page 54
at lines 21-35)**

Propagation linkers:

FokI: 5'-----GGATG
3'-----CCTACNNNN
Bst7II: 5'-----GCAGC
3'-----CGTCGNNNN
HgaI: 5'-----GACGC
3'-----CTGCGNNNN[SEQ ID NO:79]
SplI: 5'-----GAG-----CTCNNNN
3'-----CTC-----GAG
BaeI: 5'-----CCATG----CANNNNN
3'-----GGTAC----GT
HaeIV: 5'-----GAC-----GTCNNNNNN
3'-----CTG-----CTG
CjeI: 5'-----CCA-----GTNNNNNN
3'-----GGT-----CA

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Marked-up copy of the amended specification (paragraph on page 55 at lines 28-36)

The 3'-GAGTGC overhang is then ligated with the X=3 initiation linker and the GTGAA-3' overhang is ligated with the CACTT-3' overhang on the target DNA molecule:

```
5'--GCAGCGACCATGAGTCCA-CTC--GTGGATG---PPPPPP[SEQ ID NO:15]
3'--CGTCGCTGGTACTCAGGT-GAG--CACCTAC---QQQQQQ[SEQ ID NO:85]
-----GTGAA-----3'
-----CACTT-----5'
```

Marked-up copy of the amended specification (paragraphs on page 56 at line 15-page 58 at line 7)

Method 1

Two IIS enzymes that generate 5'-4 base overhangs (*Bbs*I and *Esp*3I):

```
5'..VVVVVVVVGAGC-GAGACG-----GAAGAC--GAGCIIIIIIIIII 3'[SEQ ID
NO:86]
3' VVVVVVVVCTCG-CTCTGC-----CTTCTG--CTCGIIIIIIIIII..5'[SEQ ID
NO:87]
```

After cleavage with *Bbs*I and *Esp*3I:

```
..VVVVVVVV      +      GAGC-GAGACG-----GAAGAC--[SEQ ID NO:88]      +
VVVVVVVVVCTCG      -CTCTGC-----CTTCTG--CTCG[SEQ ID NO:89]
GAGCIIIIIIIIII
```

After ligation with T4 DNA ligase:

..VVVVVVVVGAGCIIIIIIIII[SEQ ID NO:90]
VVVVVVVVCTCGIIIIIIIII..[SEQ ID NO:91]

One IIS enzyme that generates two 3' 3 base overhangs (*BsaXI*):

After cleavage with *Bsa*XI:

After ligation with T4 DNA ligase:

-----AC-----CTCC-----GAG[SEQ ID NO:94] +
CTC-----TG-----GAGG-----[SEQ ID NO:95]

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..VVVVVVVVGAGIIIIIIIIII
VVVVVVVVVCTCIIIIIIIIII..

Method 3

One IIS enzyme that generates blunt ends (*MlyI*):

5'..VVVVVVVV-----GAGTC-----IIIIIIIIII 3' [SEQ ID
NO:96]
3' VVVVVVVV-----CTGAG-----IIIIIIIIII..5' [SEQ ID
NO:96]

After cleavage with *MlyI*:

..VVVVVVVV + -----GAGTC-----[SEQ ID NO:97] +
VVVVVVVV -----CTGAG-----[SEQ ID NO:97]

IIIIIIIIII
IIIIIIIIII..

After ligation with T4 DNA ligase:

-----GAGTC-----[SEQ ID NO:97] +
-----CTGAG-----[SEQ ID NO:97]

..VVVVVVVVIIIIIIIIII
VVVVVVVVIIIIIIIIII..

**Marked-up copy of the amended specification (paragraph on page 71
at line 14-page 72 at line 4)**

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Based upon the overhang pairs, a set of five library components was made by annealing complementary oligonucleotides in separate tubes:

signal 1:

5'-TAATACGACTCACTATAACCACAAGTTTGTACAAAAAGCAGGCTCTATTC-3' [SEQ ID NO:22]

and

5'-TAGGAAGAATAGAGCCTGCTTTTTTGTACAAACTTGTGGTATAGTGAGTCGTATTA-3' [SEQ ID NO:23];

signal 2:

5'-TTCCTATGCAGTGGACCACTTTGTACAAGAAAGCTGGGTTGCAGT-3' [SEQ ID NO:24]

and 5'-GCAACTACTGCAACCCAGCTTTCTTGTACAAAGTGGTCCACTGCA-3' [SEQ ID NO:25];

signal 3:

5'-AGTTGCTTGACGCCACAAGTTTGTACAAAAAGCAGGCTTTGACG-3' [SEQ ID NO:26]

and 5'-CGACATCGTCAAAGCCTGCTTTTTTGTACAAACTTGTGGCGTCAA-3' [SEQ ID NO:27];

signal 4:

5'-ATGTCGAAGGGCGGACCACTTTGTACAAGAAAGCTGGGTAAGGGC-3' [SEQ ID NO:28]

and 5'-GACAGGGCCCTTACCCAGCTTTCTTGTACAAAGTGGTCCGCCCTT-3' [SEQ ID NO:29];

signal 5:

5'-CCTGTCATGTGGACCACTTTGTACAAGAAAGCTGGGTTTCTATAGTGTCACCTAAATC-3' [SEQ ID NO:30] and

5'-GATTTAGGTGACACTATAGAAACCCAGCTTTCTTGTACAAAGTGGTCCACAT-3' [SEQ ID NO:31];

T7: 5'-TAATACGACTCACTATACCA-3' [SEQ ID NO:32];

T7-CyS primer: 5'-TAATACGACTCACTATA-3' [SEQ ID NO:33]; and

SP6 primer: 3'-AAGATATCACAGTGGATTTAG-5' [SEQ ID NO:34].

The library components (4 pmol each) were then mixed together and ligated using 100 U T4 DNA ligase (NEB) in 1X ligase buffer at 25

Marked-up copy of the amended specification (paragraph on page 73 at lines 10-26)

Oligonucleotides are selected which bind to the fragment chain and also serve as primers. Thus for example, for adjacent chains may be bound using for example the following primer pairs:

3'AAGATATCACAGTGGATTTAGCCTACCAGTACATCCAACGGCAACT5' [SEQ ID NO:36]

3'ATTATGCTGAGTGATATCGT5' [SEQ ID NO:38]

Marked-up copy of the amended specification (paragraph on page 75 at lines 12-18)

3'...CGACCTCCGGAGGTGATACTTTAGCGCATC.....[SEQ ID NO:98]

Marked-up copy of the amended specification (paragraph on page 76 at line 1-page 77 at line 2)

5'TAA TAC GAC TCA CTA TAG CA3'[SEQ ID NO:46]

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Termination linker 1 (s):

5'AAG AGC TCA GGT CAT TGA CGT AGC TAT GAA3' [SEQ ID NO:47]

Termination linker 1/2 (as):

5'AGC TAC GTC AAT GAC CTG AG3' [SEQ ID NO:48]

Termination linker I (short version):

5'AAG AGA TGA A3' [SEQ ID NO:49]

Termination linker 2 (s):

5'ACC GCT CAG GTC ATT GAC GTA GCT TCA TT3' [SEQ ID NO:50]

Termination linker 2 (short version):

5'ACC GTC ATT3'

METHODS OF CLONING AND PRODUCING FRAGMENT CHAINS WITH READABLE INFORMATION CONTENT

The present invention relates to new methods of attaching first and second nucleic acid molecules, particularly methods of cloning in which adapter molecules mediate the binding between the first and second molecules, the resultant nucleic acid molecules thus formed and methods of generating DNA with a readily readable information content and kits for performing such methods.

Presently known cloning methods generally involve the use of restriction enzymes which are used to generate fragments for insertion and cleave vectors to produce corresponding and hence complementary terminal sequences. Generally, the enzymes which are used cut palindromic sequences and thus produce identical overhangs. Different sequences that are cut with the same restriction endonucleases can then be ligated together to form new, recombinant nucleic acids.

However, such methods suffer from a number of limitations. One disadvantage in using endonucleases that form two identical overhangs is the formation of different products on ligation. If for example two fragments A and B are to be ligated, as a consequence of common overhangs the products A+A and B+B as well as the desired A+B will be produced. Other by-products resulting from other fragments produced when A and B were formed will also be generated, e.g. reassociation into the original positions. It is therefore normal to use a separation process using agarose gels. The separation procedure however often results in a considerable loss of DNA.

Such methods necessarily suffer from various limitations including the by-products mentioned above, and the need to identify the desired end-products, e.g. if only a particular insert is to be cloned.

Other cloning techniques have been used in which

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cloning has been performed using PCR techniques, e.g. in which the PCR primers have IIS enzyme recognition sites. However, the use of PCR is disadvantageous in cloning techniques as it is time consuming and requires
5 purification steps which result in significant loss of yield. The PCR reaction may also introduce point mutations and the like and the length of the fragment is limited to the polymerase capacity, e.g. a maximum of approximately 50kb.

10 It has now surprisingly been found that by generating fragments with unique single stranded regions and then mediating the binding between a first and second nucleic acid molecule, many of these disadvantages may be avoided. In this method,
15 restriction nucleases are used that form non-identical overhangs, e.g. type IP or IIS restriction endonucleases. As will be appreciated, if one uses a restriction endonuclease that makes overhangs of 4 base pairs, each fragment that is formed will have two
20 overhangs of 4 base pairs each. It is theoretically possible therefore that 4^8 (ie. 65,536) fragments may be formed with different combinations of the two overhangs. Thus, as a rule, each fragment formed on cleavage will have a unique pair of overhangs even when cleaving large
25 nucleic acid molecules.

These unique overhangs may then be addressed and adjusted appropriately using adapters with two overhangs. For example in a cloning technique one of the overhangs is made to correspond to the overhang on
30 the insert and the other overhang is made to correspond to the overhang on the vector into which the insert is to be introduced. This method is outlined in Figure 1. In that case the DNA molecule containing the insert is cut with a restriction endonuclease which makes an
35 overhang on each side of the insert. Each of the many fragments which are formed have different overhangs such that the two overhangs at either end of the insert are

unique. Ligase is then added to bind two adapters with corresponding single stranded regions. This leads to the formation of two new overhangs at the termini of the insert, which are selected such that they can be used to bind to the vector into which the insert is to be cloned. Providing identical overhangs are not created on other molecules only the desired insert will be ligated to the adapters. In the final step the insert is ligated into the vector which has two overhangs which complement the adapters' overhangs. The overhangs in the vector may be constructed using the same principles as described for the insert.

Thus in this new method, an adapter molecule is used which is complementary to a single stranded region generated on the first nucleic acid molecule and therefore binds to that molecule, but has a different single stranded region at its other terminus, thus effectively modifying the single stranded region presented for binding by the first nucleic acid molecule fragment. The adapter's free single stranded region may then mediate the binding of the first nucleic acid molecule fragment to a second nucleic acid molecule exhibiting a complementary single stranded region.

This method of mediation has particular applications for effectively identifying and selecting a first nucleic acid molecule fragment and then mediating its binding to a second nucleic acid molecule where this was not previously possible.

Of particular relevance to methods of cloning is the generation of fragments for cloning which have different single stranded regions at their termini relative to other fragments, which may then be selected and cloned into an appropriate vector. As described herein, such fragments are generated by the use of enzymes which cleave outside their recognition site and thus produce overhangs that depend on the sequence surrounding the recognition site which is likely to vary

from fragment to fragment.

Such techniques may be used to direct only a single fragment to a particular vector or may be used to direct different fragments to different sites or indeed
5 different vectors, even within the same reaction mix, providing appropriate adapters are constructed.

These methods have particular advantages over prior art methods. In particular, the whole procedure may be carried out in one or two steps, e.g. cutting and
10 ligating simultaneously or cutting and ligating separately. Even in instances where the procedure is performed in two steps, it will often be possible to perform both steps in the same buffer, e.g. since T4 DNA ligase is known to work well in most buffers for
15 restriction endonucleases. Time- and resource-consuming precipitation procedures may therefore be avoided. Moreover, ligations can be performed with overhangs of 4-6 bases, unlike conventional cloning where overhangs of 0-4 bases are used, thereby increasing ligation
20 efficiency considerably.

Furthermore, the need to carry out gel separations may be avoided. The quantity of DNA required initially can be reduced substantially. Mutation of DNA molecules on UV exposure, a common occurrence in gel separation,
25 may also be avoided. Furthermore, laboratory staff are not exposed to carcinogenic EtBr. Also, separation problems which can occur when restriction cleavage results in fragments of similar size may be avoided. The frequency of undesirable side-products such as empty
30 vectors, too many inserts or incorrect orientation of the inserts may also be avoided.

Since it is generally not problematic if the insert is cleaved, a small selection, e.g of type IIS or Ip restriction endonucleases could provide far more cloning
35 possibilities than a corresponding selection of ordinary type II restriction endonuclease used for conventional cloning procedures. Having a few type IIS, IP and

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similar restriction endonucleases that cleave with high frequency allows for many cloning possibilities.

In the specific instance of cloning of large DNA molecules (e.g. genomic DNA) or a solution containing many different DNA molecules in parallel (e.g. a cDNA library) it is very difficult to use conventional methods. If for example a large DNA molecule is cleaved with *EcoRI*, a large number of fragments may be formed with the same overhang, and in addition a considerable proportion of these fragments may be of roughly the same size. This may lead to the formation of a large number of undesired ligation products, even with gel separation. Moreover, gel separation can be difficult if the insert is large. Furthermore, it is also often difficult, or even impossible, to find restriction endonucleases that will not cut large inserts. These problems may be reduced/eliminated using the cloning procedure described herein.

If necessary, it is possible to increase the number of base pairs in the overhangs to (e.g.) 6 by using *CjeI* or similar endonucleases to form an even greater number of possible variables and thus increase the probability of producing unique overhangs.

The advantages of the method of the invention are even greater in complex cloning procedures. If several adapters are used for example, it is possible to clone many different inserts into one and the same vector at a corresponding number of different sites in one and the same reaction, as described hereinafter in more detail.

Deletions of small or large fragments may also be achieved using the same basic principle. This opens up the possibility of making complex recombinations of *inter alia* genomic DNA (removal of endogen viruses in genomes to be used for xenotransplantation, the insertion of a large number of genes from other genomes, new combinations of genes etc.). The method can also be used for exon-shuffling and other recombinations that

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are relevant in connection with artificial evolutionary systems.

Thus, in a first aspect, the present invention provides a method of attaching a fragment of a first nucleic acid molecule to a second nucleic acid molecule, wherein said method comprises at least the steps:

- 1) cleaving said first nucleic acid molecule with a nuclease which has a cleavage site separate from its recognition site to create at least one fragment of said first nucleic acid molecule having a single stranded nucleotide region (SS1a) at at least one terminus of said fragment,
- 2) if necessary generating a single stranded nucleotide region (SS2) at at least one terminus of said second nucleic acid molecule,
- 3) binding to at least one single stranded region of step 1) (SS1a) an adapter molecule comprising at one terminus a single stranded region (SSA1) complementary to the single stranded region of said first nucleic acid molecule fragment (SS1a) and additionally comprising at the other terminus a further single stranded region (SSA2) complementary to the single stranded region (SS2) at one terminus of said second nucleic acid molecule,
- 4) ligating said adapter to said first nucleic acid fragment,
- 5) binding said adapter to said second nucleic acid molecule, and
- 6) ligating said adapter to said second nucleic acid molecule.

As used herein, said first and second nucleic acid molecules are any naturally occurring or synthetic polynucleotide molecules, e.g. DNA, such as genomic or cDNA, PNA and their analogs, which are double stranded and in which single stranded regions may be generated.

Fragments of the first nucleic acid molecule are generated by use of a nuclease which cleaves outside its

"Complementary" as used herein refers to specific base recognition via for example base-base complementarity. However, complementarity as referred to herein includes pairing of nucleotides in Watson-Crick base-pairing in addition to pairing of nucleoside

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analogs, e.g. deoxyinosine which are capable of specific hybridization to the base in the nucleic acid molecules and other analogs which result in such specific hybridization, e.g. PNA, DNA and their analogs.

5 Complementarity of one single stranded region to another is considered to be sufficient when, under the conditions used, specific binding is achieved. Thus in the case of long single stranded regions some lack of base-base specificity, e.g. mis-match, may be tolerated, 10 e.g. if one base in a series of 10 bases is not complementary. Such slight mismatches which do not affect the ultimate binding and ligation of the single stranded regions are considered to be complementary for the purposes of this invention. The single stranded 15 regions may retain portions, on binding, which remain single stranded, e.g. when overhangs of different sizes are employed or the complementary portions do not comprise all of the single stranded regions. In such cases, as mentioned above, providing binding can be 20 achieved the single stranded regions are considered to be complementary. In those cases, prior to ligation, missing bases may be filled in e.g. using Klenow fragment, or other appropriate techniques as necessary.

"Adapters" as referred to herein are molecules 25 which adapt the first nucleic acid molecule fragment for binding to a second or third nucleic acid molecule. Adapter molecules comprise at least two regions. A first portion containing a single stranded region which is complementary to the single stranded region on the 30 first nucleic acid molecule fragment and a second portion containing a single stranded region which is complementary to the single stranded region on the second nucleic acid molecule. The single stranded regions are as described hereinbefore and are preferably 35 on different strands making up the adapter molecule. The above mentioned portions are at least as large as the single stranded regions, e.g. 4 to 6 bases in

length, although they may be longer, e.g. up to 20 bases in length.

A linking region between these single stranded regions is required for the stability of the molecule. Conveniently this comprises a double stranded nucleic acid fragment, especially in methods of cloning where amplification, replication and/or translation are to be performed. However, this portion may be substituted by any appropriate molecule depending on the end use of the resulting ligated molecule. Clearly, to achieve ligation between the first and second nucleic acid molecules appropriate attachment points and moieties for ligation must be provided.

The linking portion may serve more than just a linking function and may for example provide sequences appropriate for primer or probe binding, e.g. for amplification or identification, respectively, or may contain integration sites for mobile elements such as transposons and the like. Depending on how the method is performed, the adapters preferably do not contain restriction sites for any restriction enzymes used in the method of the invention thus avoiding the need to inactivate or remove the enzymes prior to the addition of the adapters.

Conveniently adapter molecules may be exclusively comprised of a nucleic acid molecule in which the various properties of the adapter are provided by the different regions of the adapter.

Conveniently adapters are made up of two complementary oligonucleotides having between 10 and 100 bases each, e.g. between 20 and 50 bases.

In the method described above, preferably at least one first nucleic molecule fragment is generated having a single stranded region at either end (SS1a and SS1b) to each of which an adapter binds.

Preferably the method described herein is used for cloning. Thus, in the method described above, an

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adapter is bound at either end of the first nucleic acid molecule fragment (in which the adapters may be the same or different), and the unbound end of the first adapter is bound to the second nucleic acid molecule and the unbound end of the second adapter binds either to the second nucleic acid molecule (ie. at the other end distal to the binding of the first adapter, thereby forming a circular molecule) or binds to a third nucleic acid molecule. The first of these two alternatives may arise through cleavage of a circular vector to give rise to the second nucleic acid molecule to which the [adapter 1]:[first nucleic acid molecule fragment]:[adapter 2] insert is bound to re-circularize the vector. Alternatively, a linear or circular vector may be cleaved giving rise to two or more discrete fragments (herein the second and third nucleic acid molecules) which may be joined by the adapter 1:first nucleic acid molecule:adapter 2.

Thus, in a preferred feature, a first nucleic acid molecule fragment is generated which has a single stranded nucleotide region at either terminus (SS1a and SS1b), each of which is bound by an adapter, which may be the same or different, and the first of said adapters is bound to said second nucleic acid molecule and the second of said adapters binds either to said second nucleic acid molecule or to a third nucleic acid molecule.

Thus, alternatively stated, in a preferred embodiment, the present invention provides a method of cloning a fragment of a first nucleic acid molecule into a second nucleic acid molecule, wherein said method comprises at least the steps:

1) cleaving said first nucleic acid molecule with a nuclease which has a cleavage site separate from its recognition site to create one or more fragments of said first nucleic acid molecule, wherein at least one fragment has a single stranded nucleotide region at both

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termini (SS1a and SS1b),

2) cleaving said second nucleic acid molecule to create at least two single stranded regions (SS2a and SS2b) at the site of said cleavage (e.g. linearizing a circular vector or producing fragments in a linear or circular vector),

3) binding to one of the single stranded regions of step 1) (SS1a)

a first adapter molecule comprising at one terminus a single stranded region (SSA1) complementary to the single stranded region of said first nucleic acid molecule fragment (SS1a) and additionally comprising at the other terminus a further single stranded region (SSA2) complementary to one of the single stranded regions (SS2a) produced by cleavage of said second nucleic acid molecule, and

binding to a second single stranded region of step 1) (SS1b)

a second adapter molecule as defined above which binds to the second single stranded region of said first nucleic acid molecule fragment (SS1b) and to the second single stranded region (SS2b) produced by cleavage of said second nucleic acid molecule,

4) ligating said adapters to said first nucleic acid fragment,

5) binding said adapters to said second nucleic acid molecule or fragments thereof, and

6) ligating said adapters to said second nucleic acid molecule or fragments thereof.

In instances in which cleavage of the second nucleic acid molecule results in the production of two or more discrete fragments which become ligated to the first nucleic acid molecule fragment via the adapters, said fragments constitute second and third nucleic acid molecules of the invention.

Preferably, to prevent concatemirisation of [adapter:first nucleic acid fragment:adapter] units, the

Alternatively, during the method of the invention, adapters may be modified to provide single stranded portions, e.g. by the use of restriction enzymes or other appropriate techniques during the course of the reaction. Conveniently, to simplify the number of

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steps, the enzymes used to generate single stranded regions in the first, second or third nucleic acid molecules (where necessary) may be used to generate the adapter single stranded regions.

5 As mentioned previously, the single stranded region
may be 4 or more bases in length. When using longer
overhangs or where the sequence of the full
corresponding single stranded region of the first,
second or third nucleic acid molecules is not known or
unclear, a family of adapters with one or more
0 degenerate bases in the single stranded region may be
used, for example using methods to create libraries of
adapters. Degenerate bases may also be used at
positions prone to mis-match ligations.

15 For convenience a universal library of adapters may
be created for use in the method of the invention. Thus
for example, 16 different adapters with a 4 base-pair
overhang consisting of two random bases (NN) and two
bases specific to each adapter (e.g. AA, CC,...TT) may
20 be created. In this way sufficient adapters may be
created which are capable of distinguishing between 16
different first molecule fragment overhangs, which would
suffice for many cloning purposes. Similarly a library
of second molecule, e.g. vector overhangs may be
25 created.

To increase the number of permutations in an adapter library, two separate oligonucleotide libraries may be generated, one with single stranded oligonucleotides with regions that will correspond to the single stranded region of the first nucleic acid molecule fragment and the second library with single stranded oligonucleotides with regions that will correspond to the single stranded region of the second nucleic acid molecule (e.g. vector). However in common in each member of the library is a complementary region, such that when one member from the first library is selected and combined with a member of the second

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library, they will hybridize leaving free the relevant single stranded regions. Thus for example to generate an adapter with an AA overhang and a TC overhang to bind to the first and second nucleic acid molecules
5 respectively, members of the different libraries such as GGGCCCCNNAA may be combined with TCNNNCCGGGG to form:

GGCCCCCNNAA,

TCNNNCCGGGG

10 which exhibits the appropriate overhangs. When using only two 16 member libraries this allows the production of 256 different adapters.

In generating appropriate adapters conveniently the amount of mis-match which needs to be tolerated when binding to overhangs on first, second and/or third
15 nucleic acid molecules should be reduced. This may conveniently be achieved by selecting oligonucleotides on the basis of the probability of a mismatch ligation being generated. A computer program for achieving this is described in more detail in Example 6. This method
20 allows sets of oligonucleotides to be identified which can be used to construct chains with more than 100 fragments in a single ligation cycle but with very low levels of mis-match. Thus in a further feature the present invention provides computer software adapted to
25 identify adapter molecules for use in the method of the invention.

As mentioned above, the production of fragments of said first nucleic acid molecule is achieved using a nuclease which has a cleavage site separate from its
30 recognition site. In so doing, unique overhangs are created which reflect the sequence of that molecule. In a preferred feature, said nuclease is a class IP or IIS restriction enzyme or functional derivatives thereof. Such enzymes include enzymes produced synthetically
35 through the fusion of appropriate domains to arrive at enzymes which cleave at a site distal to their recognition site.

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These enzymes exhibit no specificity to the sequence that is cut and they can therefore generate overhangs with all types of base compositions. Cleavage with IIS enzymes result in overhangs of various lengths, e.g. from -5 to +6 bases in length. Preferably for performing the method of the invention, enzymes are chosen which generate 3-6, e.g. 4 base pair overhangs. Preferred enzymes for use in the invention include enzymes which produce 4 base overhangs at the 3' end:

BstXI; 5 base overhangs at the 3' end: *AloI*, *BaeI*, *BpII*, *Bsp24I*; 6 base overhangs at the 3' end: *CjeI*, *CjePI*, *HaeIV*; 4 base overhangs at the 5' end: *AceIII*, *Acc36I*, *Alw26I*, *AlwXI*, *Bbr7I*, *BbsI*, *BbvI*, *BbvII*, *Bvb16II*, *Bli736I*, *BpiI*, *BpuAI*, *BsaI*, *Bsc91I*, *BseKI*, *BseXI*, *BsmAI*, *BsmBI*, *BsmFI*, *Bso31I*, *Bsp423I*, *BspBS31I*, *BspIS4I*, *BspLU11III*, *BspMI*, *BspST5I*, *BspTS514I*, *Bst12I*, *Bst71I*, *BstBS32I*, *BstGZ53I*, *BstTS5I*, *BstOZ616I*, *BstPZ418I*, *Eco31I*, *EcoA41*, *EcoO44I*, *Esp3I*, *FokI*, *PhaI*, *SfaNI*, *Sth132I*, *StsI*; and 5 base overhangs at the 5' end: *HgaI*

Over 100 classes of IIS restriction endonucleases have been identified and there are large variations both with respect to substrate specificity and cleaving pattern. In addition, these enzymes have proved to be well suited to "module swapping" experiments so that one can create new enzymes for particular requirements (Huang-B, et al.; J-Protein-Chem. 1996, 15(5):481-9, Bickle, T.A.; 1993 in Nucleases (2nd edn), Kim-YG et al.; PNAS 1994, 91:883-887). In these experiments the binding domain of transcription factor *Sp1* was merged with the cleavage domain of *FokI* to construct a class IIS restriction endonuclease that makes a 4-base overhang with *Sp1* sites. In other experiments a class IIS restriction endonuclease that cuts outside the binding sites of transcription factor Ultrabithorax was generated. Corresponding experiments have been conducted on class I enzymes. By merging the N-terminal part of the *hsdS* sub-unit of *StyR* 1241 (which recognizes

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Generation of the single stranded regions on said first nucleic acid fragment may be achieved directly by cleavage of said first nucleic acid molecule with nucleases described herein without the development of intermediate molecules. This forms a preferred feature of the invention. Alternatively, indirect and more elaborate techniques may be used. For example, the first nucleic acid molecule or a fragment thereof may be "trimmed" using the nucleases described herein, in which linker molecules which carry the nuclease recognition site are bound to the first nucleic acid molecule or fragment thereof, and cleavage outside the recognition site results in cleavage within the first nucleic acid molecule or fragment thereof. This method is particularly useful since it takes advantage of the fact that T4 DNA ligase (and also other ligases) works well in most buffers used for restriction cutting. Ligation and cleavage can therefore be performed simultaneously in the same solution. Furthermore, this methods allows the generation of a unique overhang when the overhang generated by the first cleavage step is not unique.

The trimming procedure may be initiated using an "initiation linker" that is addressed to an overhang on the first nucleic acid molecule or fragment thereof, e.g. after cleavage with one or more restriction endonucleases as described herein. As used herein, a

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"linker" refers to a molecule which is similar to an "adapter" as described herein, except that the linker need only contain one single stranded region to allow binding to the molecule to be trimmed. Furthermore, the initiation linker contains one or more cleavage sites for nucleases that cleave outside their own recognition sequence, as described herein, for example *Bp1I*. The first nucleic acid molecule or fragment thereof should preferentially not contain cleavage sites for the IIS enzymes(s) used for the trimming procedure. Such cleavage sites may alternatively be inactivated prior to the trimming procedure (e.g. by methylation).

Propagation linkers (if used) and a termination linker (wherein the latter may be an adapter as described herein), T4 DNA ligase and the IIS enzyme(s) used for the trimming may be added together with the initiation linker. Once the initiation linker has been ligated into position, cleavage may be effected resulting in the generation of an overhang within the first nucleic acid molecule or fragment thereof. If desired (ie. if further trimming is required), a propagation linker containing degenerate overhangs may be used to ligate with the overhang which has been generated. Since the linker will also carry an appropriate nuclease recognition site, cleavage will again produce a further cleavage site further upstream into the first nucleic acid molecule or fragment thereof. This process will continue until an overhang is generated that is complementary to one of the overhangs in the termination linker (or adapter as described herein). This final linker will not itself have the nuclease recognition site and will therefore terminate trimming. As mentioned previously, this terminator linker may have an appropriate single stranded region for binding to the adapter used in the next step, or may itself be the adapter. An appropriate technique for performing the trimming method may be

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found in Examples 4 and 9.

5 The trimming method is preferably not performed with IIS enzymes belonging to the *BcgI* class (e.g. *BpII*, *BaeI* etc.) as the proteins are combined methylases and endonucleases and the methylase function may inactivate the binding sites on propagation linkers. Enzymes including *FokI*, *HgaI* etc. are therefore preferred enzymes for performing this method. If *BcgI* class enzymes are to be used, the cofactor AdoMet should be replaced with AdoHcy, Sinefungine or other cofactors that can not function as methyl donors.

10 Thus in a preferred feature the invention provides a method of removing the end terminus of a double stranded nucleic acid molecule with at least one single stranded region, comprising at least the steps of (i) binding (ie. ligated) a double stranded linker molecule containing a recognition site for a nuclease which cleaves outside its recognition site and a single stranded region complementary to the single stranded region on said double stranded nucleic acid molecule to said molecule and cleaving using said nuclease, thereby resulting in removal of one or more bases (e.g. 3-10, which may be in single or double stranded form, or a combination thereof) from the terminus of said nucleic acid molecule, (ii) optionally binding one or more propagation linkers which contain a recognition for a nuclease as described above and a degenerate single stranded region which binds to the overhang generated by the first or subsequent cleavage steps and cleaving using said nuclease, and (iii) adding a termination linker which binds to the single stranded region generated in steps i or ii.

35 A similar technique may be used to remove unwanted sequences, e.g. contributed by the adapter after ligation of the first nucleic acid molecule fragment and second (or third) nucleic acid molecules. Various techniques may be used to remove the unwanted sequences,

Depending on how the different steps in the method of the invention are performed, as described hereinafter, where necessary the second nucleic acid molecule, and/or the adapters may also be cleaved or digested to provide appropriate single stranded regions. In a preferred feature, the second nucleic acid molecule and/or the adapters are cleaved using the nucleases described above for generating the first nucleic acid molecule fragments. However, instead of cleavage with such nucleases, to generate appropriate single stranded regions and/or fragments from the second or third nucleic acid molecules or adapters, alternative techniques may be used. Thus for example other

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restriction enzymes, non-specific nucleases or appropriate exonucleases or mechanical methods such as sonication or vortexing may be used. Where enzymes are employed, small volumes are preferably used during the reactions to increase efficiency.

Ligation between the adapters and first, second and third nucleic acid molecules is achieved by any appropriate technique known in the art (see for example, Sambrook et al., in "Molecular Cloning: A Laboratory Manual", 2nd Ed., Editor Chris Nolan, Cold Spring Harbor Laboratory Press, 1989). For example, ligation may be achieved chemically or by use of appropriate naturally occurring ligases or variants thereof. Appropriate ligases which may be used include T4 DNA ligase, and thermostable ligases, such as Pfu, Taq, and TTH DNA ligase. Ligation may be prevented or allowed by controlling the phosphorylation state of the terminal bases e.g. by appropriate use of kinases or phosphatases. Appropriately large volumes may also be used to avoid intermolecular ligations. Thus, high adapter to vector/insert ratios may be used to avoid the vector or insert religating into its source material.

Other techniques may be used to avoid or remove vectors which become religated or which do not cleave. For example the insert may be cloned into a selection marker that destroys the host bacteria unless it has been inactivated by the insert. Alternatively restriction cleaving using restriction enzymes specific for the fragment removed from the vector may be performed after the ligation step. Religated and uncleaved vectors would be cleaved in this step. Thus, the ideal cloning site is therefore one which contains many unique restriction sites that are removed upon insert ligation. Alternatively well-known techniques may be used for identifying the desired product, e.g. gel separation.

If the steps of cleavage and ligation are performed

together, advantageously the insert and the vector into which it is inserted do not contain binding sites for the nuclease used. Similarly, it is advantageous if the fragment removed from the vector during the process of cloning contains binding sites for the nuclease. In that case, if that fragment religates with the vector it would be cleaved and thereby removed again.

Once the first and second nucleic acid molecules (and optionally third nucleic acid molecules) or fragments thereof have been covalently attached, where necessary selection of appropriate products from any side-products may be performed. Selection may be performed by any techniques known in the art. Conveniently however, labelled probes may be used to identify sequences present only in the correct product, e.g. by probing for one or more sequences formed only through the union of the correct sequences, e.g. a probe directed to the junction between the adapter and the first, second or third nucleic acid sequences. Alternatively, the correct ligation may be detected by functional properties bestowed on the product through ligation, e.g. through the completion of sequences which allow expression of a particular product once the vector has been cloned into an appropriate host. Alternatively, selection may be performed by sequencing of the products which have been obtained, e.g. after amplification and/or transformation.

Appropriate labels include any moieties which directly or indirectly allow detection and/or determination through the generation of a signal. Although many appropriate examples exist, examples include for example radiolabels, chemical labels (e.g. EtBr, TOTO, YOYO and other dyes), chromophores or fluorophores (e.g. dyes such as fluorescein and rhodamine), or reagents of high electron density such as ferritin, haemocyanin or colloidal gold. Alternatively, the label may be an enzyme, for example peroxidase or

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alkaline phosphatase, wherein the presence of the enzyme is visualized by its interaction with a suitable entity, for example a substrate.

As mentioned previously, one of the significant advantages which this method offers over known methods is the simplification of the techniques which are required. The steps described herein may be performed sequentially in separate tubes (e.g. when different enzymes are used and cross-reaction is undesirable) or in a limited number of steps. However, ideally, the reaction is performed in a single step. This can be achieved by appropriate selection of enzymes, adapters and second/third nucleic acid molecules, e.g. vectors.

Thus for example the first nucleic acid molecule may be fragmented using a particular nuclease which is also used to fragment the second nucleic acid molecule. Since the enzyme used will cleave outside its recognition site, it would be expected that the resulting single stranded regions found on both the first and second nucleic acid molecule fragments will be unrelated. However, by appropriate choice of the mediating adapters (which may also be added providing they do not have restriction sites for that enzyme, or that cleavage at those sites reveals appropriate single stranded regions), these unrelated sequences may be linked via the intermediacy of the adapters. Thus the entire reaction may be performed in a single step.

It will also be appreciated that the adapters may be used to address the first nucleic acid fragments to different second nucleic acid fragments or cleavage sites. This would therefore allow different first nucleic acid molecule fragments to be directed and ligated to a particular vector or site within a vector. Thus multiple vectors (and corresponding appropriate adapters) may be used simultaneously and take up a single first nucleic acid molecule fragment.

Alternatively, multiple fragments or copies of the

Thus in a preferred embodiment the method described herein is performed in a single step. The ligation steps (ie. adapter to first nucleic acid molecule fragment and final ligation) may however be conducted separately once association of the relevant molecules has been achieved. In a further preferred embodiment, the invention provides a method of simultaneously attaching two or more fragments of the first nucleic acid molecule to different second nucleic acid molecules (or different termini thereof). In cloning, this equates to the introducing of the two or more fragments into different sites in said second nucleic acid molecules or into different second nucleic acid molecules, e.g. into different sites within a vector or into different vectors.

Thus the present invention provides methods of the invention in which two or more fragments of the first nucleic acid molecule are attached to different second and optionally third nucleic acid molecules, or different termini thereof. In a preferred feature, methods are provided wherein one or more fragments of said first nucleic acid molecule are attached via adapters to single stranded regions in said second nucleic acid molecule resulting from different cleavage events. As a further preferred feature, methods are provided wherein one or more fragments of said first nucleic acid molecule are attached via adapters to single stranded regions in two or more second nucleic

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acid molecules.

It will be appreciated that even more complex reactions may be envisaged in which multiple first nucleic acid molecules (e.g. 2 or more, e.g. 2-10) are simultaneously cleaved in the same reaction and their fragments bound to appropriate adapters which direct them to bind to different second nucleic acid molecules, e.g. different vectors or sites in vectors.

Whilst the above described methods describe an especially simplified method, the above described effects may also be achieved by performing the method in discrete steps. This is particularly appropriate where different enzymes are used which would produce undesirable products in other molecules. Thus for example, different nuclease, such as restriction enzymes may be used to cleave the first and second nucleic acid molecules. In such cases, the molecules are cleaved separately, whereafter the enzymes are removed or inactivated before the fragments are mixed together with the adapters. Similarly, even if the same enzyme is used, if the adapters contain enzyme sensitive sites, the adapters could be appropriately modified to avoid reaction, e.g. by methylation, or the enzymes used to fragment the first and/or second nucleic acid molecules would be inactivated or removed (as mentioned above) prior to the addition of the adapters.

Conveniently, inactivation of enzymes may be achieved by incubation at at least 65°C, e.g. for 20 minutes. Alternatively, appropriate techniques employing removal of the enzymes from the reaction, use of chelators, inhibitors etc. may be used to achieve inactivation.

Once appropriate clones have been generated and selected these may be treated according to standard methods of amplification, transformation, replication, expression, sequencing, depending on the proposed application of the clones. Other aspects of the

invention thus include the nucleic acid molecule product of the method (ie. the nucleic acid molecule that is the [first nucleic acid molecule fragment]:[adapter]:[second nucleic acid molecule] product), such as cloning and expression vectors comprising that nucleic acid molecule product as well as transformed or transfected prokaryotic or eukaryotic host cells, or transgenic organisms containing a nucleic acid molecule produced according to the method of the invention.

Appropriate expression vectors include appropriate control sequences such as for example translational (e.g. start and stop condon, ribosomal binding sites) and transcriptional control elements (e.g. promoter-operator regions, termination stop sequences) linked in matching reading frame with the nucleic acid molecules of the invention. Appropriate expression systems are well known and documented in the art as well as methods for their introduction and expression in prokaryotic or eukaryotic cells or germ line or somatic cells to form transgenic animals. Appropriate expression vectors for transformation include bacteriophages and viruses, such as baculovirus, adenovirus and vaccinia viruses.

Kits for performing the methods described herein form a preferred aspect of the invention. Thus viewed from a further aspect the present invention provides a kit for attaching a first nucleic acid molecule fragment to a second nucleic acid molecule or a fragment thereof comprising at least (i) one or more adapters as described hereinbefore or means for producing such adapters, (ii) the second nucleic acid molecule and (iii) a nuclease which cleaves outside its recognition site, wherein the terminus of one of said adapters has a single stranded region complementary to a single stranded region generated on said second nucleic acid molecule after cleavage with said nuclease.

Preferably said kit comprises a library of oligonucleotides, e.g. as described herein, particularly

as described in Example 3, from which appropriate adapters may be generated. The library of oligonucleotides as described herein forms a further preferred feature of the invention. Thus for example
5 said library may comprise a plurality of oligonucleotides comprising 1) a plurality of oligonucleotides of the formula XNNNNN wherein X is one or more bases (wherein said bases are as described hereinbefore) and is invariant in all of said
10 oligonucleotides and each N is a base at the 5' end which is varied in the different oligonucleotides, ie. to produce 1024 variants, 2) a plurality of oligonucleotides of the formula X'NNNNN wherein X' is complementary to X and is invariant in all of said
15 oligonucleotides and each N is a base at the 5' end as described hereinbefore, 3) a plurality of oligonucleotides of the formula YNNNNN wherein Y, which is not the same as X, is one or more bases (wherein said bases are as described hereinbefore) and is invariant in
20 all of said oligonucleotides and each N is a base at the 3' end as described hereinbefore, and 4) a plurality of oligonucleotides of the formula Y'NNNNNN wherein Y' is complementary to Y and is invariant in all of said oligonucleotides and each N is a base at the 3' as
25 described hereinbefore.

Optionally the kit may contain other appropriate components selected from the list including ligases, enzymes necessary for inactivation and activation of restriction or ligation sites, primers for amplification
30 and/or appropriate enzymes, buffers and solutions, and a data carrier containing a computer program to assist in the selection of oligonucleotides from the above mentioned library. The use of such kits for performing the method of the invention form further aspects of the
35 invention.

The above described method may be adapted to combine multiple first, second, third etc. nucleic acid

molecules as described below. In this method multiple fragments are combined by appropriate selection of the single stranded regions which appear at their ends. This has application in the production of specific sequences for biological purposes, but has particular utility in the production of nucleic acid molecule chains in which the units making up the chains each denotes a unit of information, ie. the chains may be used to store information, as will be described in more detail below. As used herein "chain" refers to a serial arrangement of fragments as described herein. Such chains are preferably linear and include branched and unbranched fragment sequences. Thus, for example, branched DNA fragments may be used to provide chains with a branched arrangement of fragments.

To produce nucleic acid molecule chains with different unit fragments, ie. fragment chains the following method may be used. Firstly it is necessary to generate fragments which have overhangs at either end, to allow them to bind to one another. (The ultimate 3' and 5' fragments may however have an overhang at only the end which will become attached to internal fragments.) As will be described in more details below, for certain applications appropriate oligonucleotides may be derived from libraries in which the members exhibit variability in at least some of their bases. If libraries are to be produced in which the members are double stranded, it will be appreciated that the number of members in such a library could be rather high. This can however effectively be reduced by using a smaller number of smaller building blocks.

One strategy is to make two single-stranded oligonucleotides using conventional techniques. In the example described above (6 base double stranded linker and 3 base overhangs at either end), oligonucleotides having a region of 6 bases which complement each other and so allow hybridization may be used. Since not all

of the molecules are involved in the hybridization, single stranded regions extend beyond the hybridizing region thus creating single stranded regions. Conveniently the number of required library members may be reduced even further if repeat sequences appear with frequency in the fragment chain. This will be described in more detail below.

Once the appropriate double stranded chain units (ie. fragments) have been created they may be ligated together in the same solution, providing the different overhangs present on the sequences are unique.

Thus in a further aspect, the present invention provides a method of synthesizing a double stranded nucleic acid molecule comprising at least the steps of:

- 1) generating n double stranded nucleic acid fragments, wherein at least n-2 fragments have single stranded regions at both termini and 2 fragments have single stranded regions at at least one terminus, wherein (n-1) single stranded regions are complementary to (n-1) other single stranded regions, thereby producing (n-1) complementary pairs,
- 2) contacting said n double stranded nucleic acid fragments, simultaneously or consecutively, to effect binding of said complementary pairs of single stranded regions, and
- 3) optionally ligating said complementary pairs simultaneously or consecutively to produce a nucleic acid molecule consisting of n fragments.

The terms "nucleic acid molecule", "single stranded regions", "complementary", "binding" and "ligating" are as described hereinbefore.

In step 1) reference is made to (n-1) single stranded regions complementary to (n-1) "other" single stranded regions. This describes two families of single stranded regions, which together comprise 2(n-1) members, forming n-1 pairs. Thus "other" refers to single stranded regions in the second family which are

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not present in the first family.

"Contacting" as used herein refers to bring together the double stranded fragments under conditions which are conducive to association of the complementary single stranded regions. Depending on the method used, this may ultimately allow ligation of the fragments carrying those regions. It should however be noted that the fragments may be linked by methods other than ligation. For example PCR may be used with appropriate primers, e.g. pairs of primers.

Simultaneous or consecutive contacting and/or ligation refers to the possibility of adding the fragments individually or in groups to a growing chain or simultaneously adding all n fragments together, wherein ligation may be performed after each addition or once all n fragments have been combined. Preferably ligation is effected once all fragments have been combined.

"Fragments" as used herein are as defined herein before, but preferably are shorter in length. Thus fragments are preferably greater than 6 bases in length (wherein said length refers to the length of each single stranded oligonucleotide making up the fragment which may differ slightly in length from one another), e.g. between 6 and 50 bases, e.g. from 8 to 25 bases.

As referred to herein, "n" is an integer of at least 4, for example at least 10 or 100, e.g. between 25 and 200.

Preferably, as mentioned above, the fragments are generated by the use of single stranded oligonucleotides to generate appropriate double stranded molecules.

Of particular interest in such methods is the production of fragment chains that may be used to store information in the form of code which may readily be accessed.

There is currently a great need for storing information for different purposes (e.g. computer

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software, music, films, databases etc.). It has therefore been imperative to find efficient storage media, resulting in the development of CD ROMs, DVD technology etc. Nucleic acid molecules offer far more efficient methods for storing information and have several advantages over storage methods currently in use. For example, the storage capacity of nucleic acid molecules is vast. In principle, a test-tube containing DNA molecules may contain as much information as several million CD ROMs or more. Nucleic acid may be copied quickly and efficiently using natural systems which are greatly enhanced by techniques which have been developed such as PCR, LCR etc. When stored appropriately, nucleic acid molecules may be preserved for extremely lengthy periods. Naturally existing tools for manipulation of nucleic molecules are already available for processing of the molecules, e.g. polymerases, restriction enzymes, transcription factors, ribosomes etc. The nucleic acid molecules may also have catalytic properties.

Furthermore, nucleic acid molecules may be used as secure systems since they may be made such that they are not readily copied, unlike copying of current storage systems, e.g. CDs etc., which is increasingly prevalent.

Previously however, it was not possible to take advantage of the enormous potential offered by nucleic acid molecules due to the absence of any effective methods for writing DNA messages or reading DNA messages. The above described method provides methods which overcome this problem allowing the rapid synthesis of large DNA molecules and methods of rapidly and efficiently scanning those molecules to retrieve the information.

The key to effective retrieve of information encoded by the nucleic acid molecules produced according to the method described herein, is the expansion of the information providing unit in the molecule. In nature

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and in methods used previously, each base in the sequence has an individual informational content. Indeed methods have been described in which a single base may signify more than a single informational unit, e.g in binary code, the bases A="00", C="01", G="10" and T="11". Whilst this has advantages insofar as significant amounts of information can be contained in a single molecule, the system has serious drawbacks as it requires writing and reading methods in which individual bases may be attached and discriminated.

In a preferred method of the invention therefore, information units are provided which are not single bases, but are instead short sequences. The techniques described above allow the rapid production of such chains and the information may be readily accessed.

Thus units representing coded information may be generated and read. Each information unit may therefore represent an element of code, in which the code may for example be alphanumeric code or a simpler representation such as binary code. In each case it is necessary for individual elements of the code, e.g. "a", "b", "c", "1", "0" etc. to be represented by an individualized and specific sequence.

As used herein "information units" refer to discrete short sequences which represent a single piece of information, e.g. one or more (ie. combinations thereof) elements of a code.

"Elements" of code, as mentioned above, refer to the different members making up a code such as binary or alphanumeric code.

Thus, in a preferred embodiment of the method of the invention, the fragments which are linked together comprise regions representing a unit of information corresponding to one or more code elements. Preferably the code is alphanumeric. Especially preferably the code is binary. Thus for example, considering a binary system of information capture, if one wishes to produce

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chains consisting of "0", "1" fragments, appropriate sequence combinations may be attributed to "0" or "1".

Conveniently each of said one or more code elements (together) has the formula

5 $(X)_a$,

wherein

X is a nucleotide A, T, G, C or a derivative thereof which allows complementary binding and may be the same or different at each position, and

10 a is an integer greater than 2, e.g. greater than 4, for example from 2 to 20, preferably from 4 to 10, e.g. 6 to 8,

wherein $(X)_a$ is different for each one or more code elements.

15 Especially preferably, in the case of binary code, the code elements "1" and "0" may have the formulae:

"0" = $(X)_a$ and "1" = $(Y)_b$,

wherein

20 $(X)_a$ and $(Y)_b$ are not identical,

X and Y are each a nucleotide A, T, G, C or a derivative thereof which allows complementary binding and may be the same or different at each position, and

25 a and b are integers greater than 2, e.g. greater than 4, for example from 2 to 20, preferably from 4 to 10, e.g. 6 to 8.

As referred to herein, a "derivative" which is capable of complementary binding refers to a nucleotide analog or variant which is capable of binding to a nucleotide present in a complementary strand, and includes in particular naturally occurring or synthetic variants of nucleotides, e.g. uracil or methylated, amidated nucleotides etc.

30 In its simplest and preferred form, X and Y are the same at each position, e.g. "0" = GGGGGGGG and "1" = AAAAAAAA. However, repeat sequences such as $[AC]_6A$ or $[GT]_6A$ may be used. The code sequence may also have a

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functional property, e.g. it may be an integration element such as AttP1 or AttP2.

It will however be appreciated that the sequences described above may also denote more than a single code element. Thus for example the information unit may denote 2 or more code elements, e.g. from 2 to 32 element, preferably from 2 to 4 code elements. If for example binary code is considered, each information unit may refer to "01" or "00" or "11" or "10".

In the method described herein, chains comprising such features may be prepared as follows. To produce a chain with for example 8 0/1 fragments, eight "0" starting fragments with different overhangs and 8 "1" starting fragments with different overhangs are generated as illustrated in Figure 2. In this case "0" fragments consist of the sequence GGGGGGGG, although this could be replaced by other sequences. In addition the fragments are synthesized such that they have unique overhangs such that they may only be ligated at one position. Thus, the fragments for position 1 in the chain are produced such that they have an overhang which is complemented by one of the overhangs in the fragments for position 2. Thus, the position 2 fragments are synthesized such that they can bind to position 1 fragments. Similarly position 3 fragments may only bind to position 2 fragments at one of their termini and position 4 fragments at the other terminus and so forth. These fragments are stored separately. In order to build up a chain, selection is made from one of the two alternative for each position such that an appropriate binary chain is produced.

Thus, in the scheme outlined above, to produce a fragment chain which represents a chain 01001011, "0" fragments from positions 1, 3, 4 and 6 are mixed with "1" fragments from positions 2, 5, 7 and 8. If the fragments are then ligated together by adding ligase or using other ligation methods mentioned previously, the

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above described chain will be produced. As will be appreciated, this chain could also be achieved using for example only 4 fragments if the information unit carried on each fragment denoted 2 code elements.

5 It is furthermore possible to combine intermediate
fragment chains (e.g. containing at least 4 fragments)
with other fragment chains, which providing appropriate
overhangs exist at their termini may be ligated together
to form composite fragment chains. Thus, several cycles
10 could be conducted in parallel and the products
combined. In the method shown in Figure 2, the end
fragments have blunt ends, but clearly, appropriate
fragments could be used that similarly have overhangs at
the termini.

15 An appropriate technique for producing 8 fragment
chains, each containing 8 fragments which can then be
ligated together is illustrated in Figure 3. For
fragment chain 1, end fragments are used such that it is
possible for the completed fragment chain to ligate to
20 fragment chain 2 and so on. These may then be combined
to produce a 64 fragment chain. Similarly, 8 such
fragment chains may be combined to produce fragment
chains comprising 512 fragments.

As will be appreciated, as with the production of shorter chains, the step of ligation, when performed, is conveniently effected once all the fragment chains have been combined. However, the step of ligation may be performed sequentially if desired on addition of each subsequent fragment chain.

30 To combine 8 binary fragments per cycle, 16
different starting fragments are required, representing
the different "0", "1" alternatives at each position.
To make a chain of 64 fragments using two cycles, ie. to
produce 8 chains with 8 fragments which are then
35 ligated, only $16 + (4 \times 7) = 44$ starting fragments are
required. Thus, the number of different starting
fragments required reflects an almost linear increase in

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contrast to the combinations of the fragment chains which can be produced which increases exponentially with the number of cycles. As a consequence, very long fragment chains may be produced with a relatively small number of starting fragments.

Of course, as mentioned previously, intermediate chains longer or shorter than 8 may be produced. Since a large number of permutations exist in the overhang region, more starting fragments may be used thus allowing larger fragments to be built up in a single cycle. Thus, the number of cycles necessary to produce long chains may be reduced.

Small fragment chains produced according to the methods described herein may also be attached together by using variations of the techniques described herein. For example, complementary primer pairs may be used to link the various chains as described in Example 8. In this technique, amplification of the fragment chains is achieved using different primer pairs. The second primer in primer pair 1 is complementary to the first primer in primer pair 2 and the second primer in that pair is complementary to the first primer in primer pair 3 and so on. PCR reactions are then performed which produce products which in single stranded form are able to bind to one another through their complementary ends introduced by the primer pairs. These may then be ligated together.

Alternatively, fragment chains prepared by the methods described herein may be amplified with a primer which contains a restriction site to a nuclease which cleaves outside its recognition site. These amplification products are then digested with that nuclease to produce non-palindromic overhangs in the end of each fragment chain. By appropriate sequence selection (e.g. in the primer or fragments which are used) the overhangs which are generated allow the different fragment chains to be combined in order.

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In a preferred aspect therefore, the invention provides a method of synthesizing a double stranded nucleic acid molecule comprising at least the steps of:

1) generating fragment chains according to the method described hereinbefore;

2) optionally generating single stranded regions at the end of said fragment chains, wherein said single stranded regions are complementary to other single stranded regions on said fragment chains thus forming complementary pairs of single stranded regions;

3) contacting said fragment chains with one another, simultaneously or consecutively, to effect binding of said complementary pairs of single stranded regions.

Optionally said chains are ligated together, however, alternative techniques may be used to form the ultimate chain, e.g. PCR may be used as described herein.

Preferably intermediate fragment chains are between 4 and 20 fragments in length, e.g. 5 to 10, and between 5 and 50 such fragment chains are combined e.g. between 10 and 20.

Conveniently fragments to be used in the method of the invention are contained within libraries. Methods of producing the fragments which make up the library are well known in the art. For example a series of oligonucleotides may be produced which comprise two portions. A first portion which will form an overhang at one end and a second portion which will effect binding to a complementary oligonucleotide and which contains within that portion the information unit. By producing common hybridizing portions and variant overhangs, a series of double stranded oligonucleotides for one or more code elements (denoted by at least a part of the hybridizing portion) are created. This provides a library for one (or a combination of) code elements. Different libraries may be created for different code elements (or combinations thereof), by

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appropriate alteration of the information unit, ie. the sequence in the hybridizing portion.

Conveniently for use in the invention, these different double stranded oligonucleotides are arranged in 2 dimensional arrays such that in one dimension consecutive positions within the ultimate fragment are indicated and in the second dimension the possible code element (or combinations thereof) are provided. In the simplest case, in binary code, in which "0" and "1" are represented by different sequences, the first dimension would comprise fragments for each position of the proposed fragment and the second dimension would have only 2 variants ("0" and "1"). This may be viewed as a single library or two libraries, ie. the "0" or "1" libraries. Once these libraries are produced, fragment chains with any desired order of fragments may be readily produced.

In order to appropriately direct library members to their correct site or well (ie. the library may be comprised of separate solid supports, or a solid support with different addresses, e.g. wells, or different wells containing different solutions), any appropriate sorting technique may be used. This sorting may be achieved by virtue of the process used for production of the library members, or sorting may be achieved by an appropriate technique, e.g. by binding to complementary oligonucleotides at the relevant library site.

Appropriate solid supports suitable for attaching library members are well known in the art and widely described in the literature and generally speaking, the solid support may be any of the well-known supports or matrices which are currently widely used or proposed for immobilization, separation etc. in chemical or biochemical procedures. Thus for example, the immobilizing moieties may take the form of beads, particles, sheets, gels, filters, membranes, microfibre strips, tubes or plates, fibres or capillaries, made for

example of a polymeric material e.g. agarose, cellulose, alginate, teflon, latex or polystyrene. Particulate materials, e.g. beads, are generally preferred.

Conveniently, the immobilizing moiety may comprise
5 magnetic particles, such as superparamagnetic particles.

In a preferred embodiment, plates or sheets are used to allow fixation of molecules in linear arrangement. The plates may also comprise walls perpendicular to the plate on which molecules may be
10 attached. Attachment to the solid support may be performed directly or indirectly and the technique which is used will depend on whether the molecule to be attached is an oligonucleotide for fixing the library member or the library member itself. For attaching the
15 library members directly, ie. not via binding to an oligonucleotide, conveniently attachment may be performed indirectly by the use of an attachment moiety carried on the nucleic acid molecules and/or solid support. Thus for example, a pair of affinity binding
20 partners may be used, such as avidin, streptavidin or biotin, DNA or DNA binding protein (e.g. either the lac I repressor protein or the lac operator sequence to which it binds), antibodies (which may be mono- or polyclonal), antibody fragments or the epitopes or
25 haptens of antibodies. In these cases, one partner of the binding pair is attached to (or is inherently part of) the solid support and the other partner is attached to (or is inherently part of) the nucleic acid molecules. Alternatively, techniques of direct
30 attachment may be used such as for example if a filter is used, attachment may be performed by UV-induced crosslinking. When attaching DNA fragments, the natural propensity of DNA to adhere to glass may also be used.

Oligonucleotides to be used for capture of the
35 library members may be attached to the solid support via the use of appropriate functional groups on the solid support.

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Attachment of appropriate functional groups to the solid support may be performed by methods well known in the art, which include for example, attachment through hydroxyl, carboxyl, aldehyde or amino groups which may be provided by treating the solid support to provide suitable surface coatings. Attachment of appropriate functional groups to the nucleic acid molecules of the invention may be performed by ligation or introduced during synthesis or amplification, for example using primers carrying an appropriate moiety, such as biotin or a particular sequence for capture.

In a further aspect therefore the present invention provides a library of fragments as defined herein comprising $(n)_m$ fragments, wherein n is as defined hereinbefore and corresponds to the length of chain that said library may produce, and m is an integer corresponding to the number of possible code elements or combinations thereof, such that fragments corresponding to all possible code elements for each position in the final chain are provided.

Portions of said libraries in one dimension, ie. comprising n fragments for only a single code element (or combinations thereof) or comprising m fragments representing all code elements (or combinations thereof) for a single position on the chain, form further aspects of the invention.

Appropriate mixing may be achieved by automation. For example in the case of "0", "1" fragments, the correct combination of these elements is the critical step in terms of resource- and time-consumption. This method is described in more detail in Example 2. In particular, the procedure may be miniaturised providing appropriate amplifying methods (such as cloning and/or PCR) are employed in the last step. Thus, techniques using technology such as sorting using flow cytometers may be employed as described in Figure 4C. Such sorting procedures are well established and are able to sort

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approximately 5-30000 droplets per second for standard equipment, but up to 300000 droplets per second for the most advance cytometers.

As mentioned previously, it is possible that each fragment may denote more than a single code element. If for example, each fragment denotes 5 code elements, using existing technology and a library of 32x100 library components, if 3200 containers were connected to a sorting device illustrated in Figure 4C, it should be possible to write several thousand chains with 500 code elements per second. Clearly, a method which can generate nucleic acid sequences with such rapidity offers significant advantages over known methods in the art.

The nucleic acid molecule (ie. the fragment chain) produced according to the above described method and the single stranded molecules thereof comprise further features of the invention. These molecules may as appropriate be included into a vector, as described hereinbefore.

Once produced, the fragment chains, in double stranded or single stranded form, may be used in various applications, as described hereinafter. One application of particular utility is to store information. In such cases appropriate means of reading the information stored in those chains is required. In some applications, fragment chains may be appropriately addressed to particular sites, e.g. through binding to oligonucleotides carried on solid supports which are complementary to overhangs on one terminus of the fragment chains. Alternatively appropriate antibody/antigen, or DNA:protein recognition systems may be used. Thus, information stored in molecules addressed in this way, or in solution may then be accessed.

Co-pending application PCT/GB99/04417, a copy of which is appended hereto, describes appropriate

Thus in a further aspect, the present invention provides, a method of identifying the code elements contained in a nucleic acid molecule prepared as described hereinbefore (ie. fragment chain) wherein a probe, carrying a signalling means (e.g. a label), specific to one or more code elements, is bound to said nucleic acid molecule and a signal generated by said signalling means is detected, whereby said one or more code elements may be identified.

Preferably said signalling means is a label as described hereinbefore.

A "probe" as referred to herein refers to an appropriate nucleic acid molecule, e.g. made up of DNA, RNA or PNA sequences, or hybrids thereof, which is able to bind to the target nucleic acid molecule (which may be single or double stranded) through specific interactions, ie. is specific to particular code elements, e.g. through complementary binding to a particular sequence. Probes may be any convenient length, to allow specific binding, e.g. in the order of 5 to 50 bases, preferably 8 to 20 bases in length.

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A "signalling means" as used herein refers to a means for generating a signal directly or indirectly. A signal may be any physical or chemical property which may be detected, e.g. presence of a particular product, colour, fluorescence, radiation, magnetism, paramagnetism, electric charge, size, or volume. Preferably the label is a fluorophore whose fluorescence is detected. In such cases fluorescence scanners may be used for detection of the label and thereby identification of the code elements.

A particular code element or combination of elements may be identified by the appearance of a particular signal. Clearly the position of each signal is crucial to determining the sequence of the code elements. As a consequence methods in which positional information (absolute or relative) may be obtained should be used. Appropriate techniques, e.g. using target molecules which have been attached to a solid support at one end, are described in co-pending application PCT/GB99/04417.

A number of applications exist for the fragment chains once produced in nano and pico-technology, *inter alia* for example by stretching of the fragment chains by means of a stream of liquid, electricity or other technology and using them as templates for nano and pico-structures. The products may also be used to label products which can then be screened to establish their identity. Alternatively, the molecules may be used to store information, e.g. pictures, text, music or as data storage in DNA computers. The rapid production and reading techniques makes such applications possible for the first time.

Kits for performing the methods described above form a preferred aspect of the invention. Thus viewed from a further aspect the present invention provides a kit for synthesizing a double stranded nucleic acid molecule comprising at least n double stranded nucleic

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acid fragments, wherein at least $n-2$ fragments have single stranded regions at both termini and 2 fragments have single stranded regions at at least one terminus, wherein $(n-1)$ single stranded regions are complementary to $(n-1)$ other single stranded regions, thereby producing $(n-1)$ complementary pairs. Preferably in excess of n fragments are supplied for production of a chain of n fragments, such that selection of appropriate fragments for different positions is possible. Thus in a preferred feature said kit comprises $(n)_m$ fragments, wherein n is as defined hereinbefore, and m is an integer corresponding to the number of possible variations, e.g. unique sequences or code elements or combinations thereof, such that fragments corresponding to all possible sequences or code elements for each position in the final chain are provided. Preferably these fragments are provided in appropriate libraries arranged with reference to their position within the fragment chain and the code element(s) which they represent, such that desired fragments may be readily selected from the array.

Optionally the kit may contain other appropriate components selected from the list including ligases, enzymes necessary for inactivation and activation of restriction or ligation sites, primers for amplification and/or appropriate enzymes, buffers and solutions. The use of such kits for performing the method of the invention form further aspects of the invention.

The following examples are given by way of illustration only in which the Figures referred to are as follows:

Figure 1 shows a schematic representation of how the method of the invention may be used to introduce an insert into a vector, in which the insert is cleaved from the first nucleic acid molecule, associated with adapters and ligated thereto and then ligated into the

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vector;

Figure 2 shows the production of a fragment chain using 8 "0" and "1" starting fragments with different overhangs;

5 Figure 3 shows the production of a 64 fragment chain in which 8 chains are produced comprising 8 fragments each, in which the termini of chains 1 and 2, and 2 and 3 etc. are complementary such that they may be ligated together;

10 Figure 4 shows 3 techniques for mixing "0", "1" fragments from a library of fragments ordered for each position, in which in A) appropriate fragments are selected by aspiration from appropriate wells, B) appropriate fragments are released from the library
15 wells and C) a flow cytometer is used to direct appropriate droplets to the mixing chamber;

Figure 5 shows PCR amplification of signal chain 1-0-1-0-0 using SP6 and T7 primers. Lane 1: 1 μ g of 1 kb DNA ladder (Gibco BRL), Lane 2: 10 μ l of PCR amplified
20 fragment chain DNA using SP6 and T7 primers. Lane 3: Same as lane 2 except for the use of SP6 and T7-Cy5 primers; and

Figure 6 shows the use of primer pairs during the process of amplification to join together fragment
25 chains.

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EXAMPLE 1: CLONING OF AN INSERT INTO A VECTOR, FOR
EXAMPLE FROM PHIX174 INTO PUC19

A general procedure to be followed using IIS and IP enzymes to achieve cloning involves the use of a cloning vector which has the following characteristics:

- 1) A multiple cloning site located within a gene (lacZ, ccdB or other) that allows the detection of successful insertion.
- 2) The multiple cloning site contains two flanking *HgaI* sites that generates overhangs that differ from other *HgaI* generated overhangs elsewhere in the vector. The orientation of the *HgaI* sites ensures excision of its sites from the vector part during digestion. To minimize background due to undigested plasmids, several *HgaI* sites and other suitable restriction enzyme sites are included in the MCS. The restriction enzymes are chosen such that they cleave well in *HgaI* buffer and do not have other sites in the vector.

The donor plasmid is cut with the appropriate set of IIS and/or IP enzymes. Adapters are used to specify the fragment to be sub-cloned into the vector, by the use of appropriate single stranded regions on the adapters to the overhangs generated on the insert. This results in the molecule: vector - adapter 1 - insert (e.g. PhiX174 gene) - adapter II - vector.

This method is illustrated for insertion of a PhiX174 insert into a vector, e.g. pUC19. An *Hga*I site in a pUC19 plasmid is chosen randomly to be our "polylinker" while different genes and gene combinations from the PhiX174 genome is used as "inserts".

Genomes are organized in PhiX174 as illustrated below which shows the position of genes A, B, C and E relative

to one another:

```

---[-----A-----]-----
-----[----B----]-----
5  -----[---C---]-----
-----[---E---]-----
-1---2---3---4---5-----6-----7-8-----9

```

In the above, gene B is located inside gene A while gene C is slightly overlapping with gene A (by 3 base pairs). Gene D and K are located in the same area as gene C and E, but are not shown. This genome area contains 9 *BbvI* sites as shown on the bottom row, in which the overhang pairs that will be generated by cutting with *BbvI* are as follows with the base pair position indicated in brackets: 1-CAGC/GTCG (3798), 2-CTGC/GACG (4215), 3-ACGG/TGCC (4398), 3-GCAT/CGTA (4677), 5-CTAT/GATA (5049), 6-GAGA/CTCT (158), 7-GAGC/CTCG (547), 8-CAAC/GTTG (624), 9-CCAT/GGTA (892). The parts of the PhiX174 genome not shown contain 5 more *BbvI* sites: 10-TACC/ATGG (1488), 11-TACC/ATGG (1592), 12-CTAC/GATG (1639), 13-GCAC/CGTG (3294), 14-CTAA/GATT (3297). Of these only 12 give rise to non-identical overhangs whilst 2 result in identical overhangs.

When *HgaI* is used to cleave pUC19, 4 non-identical sites are cleaved, giving rise to 8 non-identical overhangs. These are: 1-CTGCC/GACGG (573), 2-TTCTC/AAGAG (1131), 3-CAAGG/GTTCC (1881), 4-AGACT/TCTGA (2459).

Method:

To sub-clone gene B from Bacteriophage PhiX174 into the designed vector, the following protocol is used:

1) 2µg of PhiX174 DNA is digested with 2 U of *BbvI* (NEB) in 1X buffer 2 (NEB), water added to a volume of 20µl, for 1 hr at 37°C. *BbvI* is then heat inactivated at 65°C

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for 20 minutes.

2) 2 μ g of vector (e.g. pUC19) is digested with 2 U *Hga*I (NEB) in 1X buffer 1 (NEB), water added to a volume of 20 μ l, for 1 hr at 37°C. *Hga*I is then heat inactivated at 65°C for 20 minutes.

3) The adapters are made in separate tubes by mixing two and two oligonucleotides (selected to obtain the desired product, ie. particular gene(s), in forward/reverse orientation) and allowing annealing.

4) 6 μ l of the cleavage reaction of *Phi*X174 is mixed with 3 μ l of the cleavage reaction of the vector and ligated in the presence of 5-50 pmol of each adaptor, 2-10 U/ μ l T4 DNA Ligase (NEB), 1X ligase buffer (NEB) and 5% Polyethylene glycol 8000, water added to a volume of 30 μ l, at 25°C for 1 hr.

5) Conventional methods are used to transform bacteria.

6) The colonies are then counted and some of them are then picked for further analysis (sequencing, and the like).

Materials:

Oligonucleotides used to address *Phi*X174 overhangs:

*Bbv*I overhang 1a:

5' - CGA GCG CCT CCA GTG CAG CGG AG

*Bbv*I overhang 5a:

5' - TATC GCG CCT CCA GTG CAG CGG AG

*Bbv*I overhang 6b:

5' - CTCT GCG CCT CCA GTG CAG CGG AG

*Bbv*I overhang 6(delC):

5' - CTCT CTC CGC TGC ACT GGA GGC GC

*Bbv*I overhang 7a:

5' - CAAC GCG CCT CCA GTG CAG CGG AG

*Bbv*I overhang 9b:

5' - GGTA GCG CCT CCA GTG CAG CGG AG

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Cloning site 1a

5' - AAGAG CTC CGC TGC ACT GGA GGC GC

Cloning site 1b

5 5' - CTCTT CTC CGC TGC ACT GGA GGC GC

Two important advantages with this recombination-method over the classical Cohen-Boyer method should be noted. The procedure is very easy to perform. It involves only mixing and incubation steps before transformation. No PCR-amplifications or gel separations are required. The methods gives significant flexibility and allows complex recombinations to be made even with only two restriction enzymes.

EXAMPLE 2: AUTOMATION AND MINIATURISATION OF CHAIN SYNTHESIS

20 This method describes a rapid process for mixing appropriate "0" and "1" fragments with the correct overhangs to produce a particular string consisting of "0"'s and "1"'s.

Two libraries are produced, one with "0" fragments and
one with "1" fragments. As mentioned in the
description, these are generated with overhangs that can
be ligated to corresponding overhangs for fragments at
adjacent positions. These separate members are present
in separate wells to form the library, such that
position 1 fragments are present in well 1, position 2
fragments are present in well 2 and so forth. The two
libraries thus provide the alternatives for each
position. In order to generate the chain therefore it
is only necessary to select the correct fragment "0" or
"1" for position 1, and then position 2 etc. Since
these fragments, as a consequence of their unique
overhangs, may only hybridize to fragments for adjacent

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positions, it is necessary only to select the correct fragments, then mix and ligate those fragments simultaneously. Different ways of achieving this effect are shown in Figure 4 which shows three different alternatives for mixing.

In Figure 4A, e.g. to produce the chain 0-1-0-0-1, the apparatus is used to aspirate from the "0" library at positions 1, 3 and 4, and aspirate from the "1" library at position 2 and 5. The liquids that have been aspirated may then be mixed together with ligase and an appropriate buffer. In alternative B, each well in the library is connected with a tube/nozzle that may be closed/opened electronically. Liquid from the nozzles is directed into the ligation chamber together with ligase and an appropriate buffer. Different chains may be constructed by appropriately changing the pattern of nozzles which are opened/closed.

The procedure may also be miniaturised, e.g. using flow cytometry technology as illustrated in Figure 4C. In this method, library components are stored in containers on top of the "writing-machine". Droplets from each container are then guided either to the waste or production well depending on the nature of the chain that is to be constructed. The guiding mechanism is as used in ordinary flow cytometers, ie. the droplets are charged when they leave the container and may be guided electronically in different directions.

EXAMPLE 3 - LIBRARIES COMPRISING OLIGONUCLEOTIDES FOR USE IN THE INVENTION

Conveniently, the cloning method may be performed using libraries containing oligonucleotides. For example a library may contain:

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1. Oligonucleotides with a common portion and 5 bases at the 5' end which vary to provide all possible permutations, ie. 1024 variants.
2. Oligonucleotides with a common portion and 4 bases at the 5' end which vary to provide all possible permutations, ie. 256 variants.
3. Oligonucleotides with a common portion and 5 bases at the 3' end which vary to provide all possible permutations, ie. 1024 variants.
4. Oligonucleotides with a common portion and 6 bases at the 3' end which vary to provide all possible permutations, ie. 4096 variants.

In the above, the oligonucleotides are produced such that all "1" oligonucleotides are complementary to "2" oligonucleotides by virtue of the invariant bases, ie. to generate a double stranded molecule with variant 4/5 base overhangs. Similarly "3" and "4" oligonucleotides are complementary.

Oligonucleotides combined in this way (ie. with overhangs at either end of 4-6 bases may also be combined together with complementary double stranded oligonucleotides also generated by combining certain members of the library. In this way variable overhangs of different lengths may be created in the resultant molecule, e.g. a molecule with a 4 base overhang at both the 3' and 5' end.

Oligonucleotides may also be provided in the library which allow 5' and 3' adapters to be linked. Thus for example oligonucleotides having the following form may be provided:

5. 5'-AAAA-[comp1]-FFFFF-3'
6. 5'-DDDDD-[comp1]-FFFFF-3'
7. 5'-AAAA-[comp1]-HHHHHH-3'
8. 5'-DDDDD-[comp1]-HHHHHH-3'

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9. 3'-[comp1*]-5'
10. 5'-BBBB-[comp2]-3'
11. 5'-EEEEEE-[comp2*]-3'
12. 5'-[comp3]-GGGGG-3'
- 5 13. 5'-[comp3*]-IIIIII-3'

in which "comp_x" refer to a region which is complementary to region "comp_x", ie. "5", "6", "7" or "8" can bind to "9". Furthermore, "comp₂" can bind to
 10 oligonucleotide 1 above, "comp_{2a}" can bind to oligonucleotide 2, "comp₃" can bind to oligonucleotide "4" and "comp₃*" can bind to oligonucleotide "3". The bases denoted "A" bind to "B", ie. "7" and "10" can bind at their ends. Similarly "D" binds to "E", "F" binds to
 15 "G" and "H" binds to "I". (These bases when together may have a variable content, e.g. AAAA=GAGA and then BBBB=TCTC.)

By appropriate use of the linkers described above, 5'
 20 and 3' adapters may be combined. For example, oligonucleotide "2" with a particular 4 base 5' overhang may be bound through its complementary region to an oligonucleotide linker "11" which will then leave a "EEEEEE" overlap. This may be bound to oligonucleotide
 25 "8" through the overlap which may itself bind oligonucleotide "9" through its complementary region. The overlap "HHHHHH" may be bound to oligonucleotide "13" which may attach an oligonucleotide "4" through binding to the complementary region. Thus various
 30 permutations may be made which result in various overlap lengths, e.g. any combination of 4, 5, or 6 base overlaps which may on the same or different strands.

35 EXAMPLE 4 - TRIMMING PROCEDURE FOR GENERATING UNIQUE OVERHANGS

The system presented here makes it possible to perform a

trimming procedure with seven different IIS enzymes that make 5' 4 base overhangs (*FokI* and *Bst71I*), 5' 5 base overhangs (*HgaI*), 3' 5 base overhangs (*BplI* and *BaeI*) and 3' 6 base overhangs (*CjeI* and *HaeIV*). If the oligonucleotide system presented here is combined with the basic oligonucleotide kit described in Example 3, all permutations of 3' 5 base and 6 base overhangs and all permutations of 5' 4 base and 5 base overhangs can be addressed for the trimming procedure.

In this Example, the location of the binding motifs of the initiation linkers is shown below:

<i>FokI</i>	-----GGATG----
<i>Bst71I</i>	--GCAGC-----
<i>HgaI</i>	-----GACGC
<i>BplI</i>	-----GAG-----CTC-----
<i>BaeI</i>	-----CYATG-----CA-----
<i>CjeI</i>	-----CCA-----GT-----
<i>HaeIV</i>	-----GAY-----RTC-----
Consensus	--GCAGCGACCATGAGTCCA-CTC--GTGGATGACGC

Initiation linkers:

X=0: 5' --GCAGCGACCATGAGTCCA-CTC--GTGGATGPPPPPP

3' --CGTCGCTGGTACTCAGGT-GAG--CACCTAC

X=1: 5' --GCAGCGACCATGAGTCCA-CTC--GTGGATG-PPPPPP

3' --CGTCGCTGGTACTCAGGT-GAG--CACCTAC-

X=2: 5' --GCAGCGACCATGAGTCCA-CTC--GTGGATG--PPPPPP

3' --CGTCGCTGGTACTCAGGT-GAG--CACCTAC--

X=3: 5' --GCAGCGACCATGAGTCCA-CTC--GTGGATG---PPPPPP

3' --CGTCGCTGGTACTCAGGT-GAG--CACCTAC---

X=4: 5' --GCAGCGACCATGAGTCCA-CTC--GTGGATGACGPPPPPP

3' --CGTCGCTGGTACTCAGGT-GAG--CACCTACTGCG

X=5: 5' --GCAGCGACCATGAGTCCA-CTC--GTGGATGACGC-PPPPPP

3' --CGTCGCTGGTACTCAGGT-GAG--CACCTACTGCG-

X=6: 5' --GCAGCGACCATGAGTCCA-CTC--GTGGATGACGC--PPPPPP

3' --CGTCGCTGGTACTCAGGT-GAG--CACCTACTGCG--

```

FokI:      5'-----GGATG
            3'-----CCTACNNNNN

Bst71I:    5'-----GCAGC
            3'-----CGTCGNNNNN

HgaI:      5'-----GACGC
            3'-----CTGCGNNNNNN

BplI:      5'-----GAG-----CTCNNNNNN
            3'-----CTC-----GAG

BaeI:      5'-----CCATG-----CANNNNNN
            3'-----GGTAC-----GT

HaeIV:     5'-----GAC-----GTCNNNNNNNN
            3'-----CTG-----CTG

CjeI:      5'-----CCA-----GTNNNNNNNN
            3'-----GGT-----CA

```

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Termination linkers:

The adapters made with the basic oligonucleotides described earlier can be used as termination linkers. There is therefore no need for a separate set of termination linkers.

Method:

In this method a trimming reaction using *Bst*71I that will begin on a 3' 5 base overhang is shown. The target DNA is shown below in which the first overhang that will be generated is marked "*".

-----*****-----
3'CACTT-----*****-----

The first *Bst*71I overhang in the target DNA will be located 5-8 bases downstream of the overhang CACTT-3'. X must therefore be 3 (see the figure below). The following strategy can then be applied:

One linker is prepared that can address the 3' GTGAA overhang by annealing 4-3' 6 bases(QQQQQQ) with 3-3' 5 bases(GTGAA) in one tube:

-----GTGAA -3'

3'- OOOOOO-----

The 3'-GAGTGC overhang is then ligated with the X=3 initiation linker and the GTGAA-3' overhang is ligated with the CACTT-3' overhang on the target DNA molecule:

5' --GCAGCGACCATGAGTCCA-CTC--GTGGATG--PPPPPP-----
3' --CGTCGCTGGTACTCAGGT-GAG--CACCTAC--QQQQQQ-----

-----GTGAA-----
-----CACTT-----

EXAMPLE 5 - REMOVAL OF INTERVENING SEQUENCES FROM
CONSTRUCTS

In some instances, constructs may be prepared which
5 contain undesirable nucleic acid sequences between, e.g
the insert sequence and the vector sequence. Strategies
for removing the linker sequences should then be
applied. Illustrated below are some possible strategies
10 in which binding sites for restriction enzymes are
provided in the adapter sequences. Cleavage with the
restriction enzymes will then result in DNA ends that
can be religated. The vector DNA is marked as ..VVVVVV
while insert DNA is marked as IIIIIII.

15 *Method 1*

Two IIS enzymes that generate 5'-4 base overhangs (*BbsI*
and *Esp3I*):

20 ..VVVVVVVGAGC-GAGACG-----GAAGAC--GAGCIIIIIIIIII
VVVVVVVCTCG-CTCTGC-----CTTCTG--CTCGIIIIIIIIII..

After cleavage with *BbsI* and *Esp3I*:

25 ..VVVVVVV + GAGC-GAGACG-----GAAGAC-- +
VVVVVVVCTCG -CTCTGC-----CTTCTG--CTCG

GAGCIIIIIIIIII
IIIIIIIIII..

30 After ligation with T4 DNA ligase:

GAGC-GAGACG-----GAAGAC- +
-CTCTGC-----CTTCTG-CTCG

35 ..VVVVVVVGAGCIIIIIIIIII
VVVVVVVCTCGIIIIIIIIII..

```
IIIIIIIIII
IIIIIIIIII..
```

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After ligation with T4 DNA ligase:

```

-----GAGTC----- +
-----CTGAG-----

```

5

```

..VVVVVVVVIIIIIIIIII
VVVVVVVVIIIIIIIIII..

```

EXAMPLE 6 - IDENTIFYING OLIGONUCLEOTIDE SETS WITH 6 BASE PAIR OVERHANGS WITH MINIMAL MIS-MATCH LIGATIONS

10

In order to identify oligonucleotide sets with 6 base pair overhangs which are unlikely to form mis-match ligations with one another the following steps may be taken.

15

1. Create all 2048 overhang pairs of 6 bases.
2. Remove the 32 palindromic pairs.

20

This produces a final set of 2016 overhang pairs.

PART 1

1. Take a pair as pair #1 and select the next pair by executing section 1.

25

Section 1

Algorithm 1

Compute the $(2016 - n)$ tables of unweighted mismatch scores between the already chosen n pair(s) and all $(2016 - n)$ remaining pairs, and find among the latter the pair(s) for which the lowest score in the table is the highest (see below for details about score computation). If there is only one such pair, then select it. If there are several pairs, then compute the weighted mismatch scores of the overhang comparisons that gave the lowest unweighted score and find the pair(s) for which the lowest weighted score is the

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highest. If there is only one such pair, then select it. If there are several pairs, then redo the whole procedure using the second lowest unweighted score in the mismatch table, then the third lowest, and so on.

- 5 If several pairs remain tied after all mismatch scores have been considered, keep them all.

Repeat algorithm 1 for each selected pair and iterate it over the desired number of positions to obtain the
10 chain(s) of overhang pairs. This procedure generates a tree with an overhang pair on each branch. The lowest unweighted and weighted mismatch scores of the particular combination of pairs at each point are computed. A particular pathway is stopped (1) when the
15 desired number of positions is reached, or (2) when the combination of pairs is one that has already been found earlier, or (3) when the lowest mismatch scores of that combination are lower than the lowest scores of the complete chain(s) already constructed. Point (3) ensures
20 that each new complete chain always has lowest mismatch scores that are higher than or at least equal to those of the previously constructed chain(s). Note also that, as a result of this process, all pairs in a given chain are unique and all complete chains in the tree are
25 unique. The whole process terminates when the last pathway to be explored stops. Keep the complete chain(s) whose lowest mismatch scores are the highest.

Repeat section 1 starting with each of the 2016 pairs as
30 pair #1 to produce a set of 2016 overhang chains. Find the best chain(s) by applying algorithm 2

Algorithm 2

For all chains, compute the tables of unweighted
35 mismatch scores between all the pairs that are present in the chain, and find the chain(s) for which the lowest score in the table is the highest (see below for

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procedure generates a tree with a chain on each branch. Each new chain which is added to the tree has a mismatch score higher than or equal to the score of the chain found in the previous step. A particular pathway is
5 stopped when the selected chain is one that has already been found earlier. This ensures that all chains in the tree are unique. The whole process terminates when the last pathway to be explored stops. Keep all the chains that are present in the tree.

10 Repeat section 2 (i.e., construct a tree) starting with each of the chains selected at the end of part 1.

From the whole set of chains present in all trees,
15 select one or more chains following algorithm 2.

This produces a final set of one or more overhang chains.

20 COMPUTATION OF MISMATCH SCORES

Unweighted score

The unweighted score for a ligation between two 6-base overhangs is the number of mismatches observed,
25 considering the triplets of the first 3 and the last 3 bases separately. For example, the score for the ligation AAAAAC/TTTGCA is 0-3 and the score for AAAAAC/TCAGGG is 2-2. All possible scores are ranked from highest to lowest according to the order below:

30 highest: : 3-3
3-2/2-3
2-2
3-1/1-3
35 2-1/1-2
1-1
3-0/0-3

The corresponding table is:

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	2B	TTTGCA	3-2	3.4
	3B	GGGACT		

Here, the lowest score is 2-2; 2.4 given by the ligation
 5 between overhangs 1A and 3B.

Score table for a chain

To compute the table of mismatch scores for a chain, all
 10 overhang pairs contained in the chain are compared with
 each other and also every overhang is compared with
 itself. Thus, for a chain of p overhang pairs, the
 number of ligations considered is $4p(p-1)/2 + 2p =$
 $2(p^2)$. As above, one of the two overhangs is reversed
 15 in the comparison when both are on the same DNA strand.

For example, let us consider the following 3-pair (i.e.,
 4-position) chain: AAAAAC/TTTTTG (1A/1B), AAACGT/TTTGCA
 (2A/2B), AGTCCC/TCAGGG (3A/3B) in which 1A is on one
 20 fragment, 1B and 2A are on a second fragment, 2B and 3A
 are on a third fragment and 3B is on a fourth fragment.

The corresponding table is:

Comparison	Overhang	Ligation	Unweighted Score	Weighted Score
1 vs 1	1A 1A	AAAAAC CAAAAA	3-3	0.8
	1B 1B	TTTTTG GTTTTT	3-3	3.2
2 vs 2	2A 2A	AAACGT TGCAAA	2-2	2.8
	2B 2B	TTTGCA ACGTTT	2-2	4.4

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	3 vs 3	3A	AGTCCC	2-2	3.6
		3A	CCCTGA		
		3B	TCAGGG	2-2	3.6
		3B	GGGACT		
5	1 vs 2	1A	AAAAAC	2-3	1.8
		2A	TGCAAA		
		1A	AAAAAC	0-3	3.8
		2B	TTTGCA		
		1B	TTTTTG	0-3	5.0
		2A	AAACGT		
		1B	TTTTTG	2-3	3.8
		2B	ACGTTT		
10	1 vs 3	1A	AAAAAC	3-2	2.6
		3A	CCCTGA		
		1A	AAAAAC	2-2	2.4
		3B	TCAGGG		
		1B	TTTTTG	2-2	4.0
		3A	AGTCCC		
		1B	TTTTTG	3-2	4.6
		3B	GGGACT		
15	2 vs 3	2A	AAACGT	3-2	2.7
		3A	CCCTGA		
		2A	AAACGT	2-2	3.3
		3B	TCAGGG		
		2B	TTTGCA	2-2	3.6
		3A	AGTCCC		
		2B	TTTGCA	3-2	3.4
		3B	GGGACT		

20 Here, the lowest score is 0-3; 3.8 given by the ligation between overhangs 1A and 2B.

Results obtained:

Table of breaking points

5 PART 1

# of positions	Unweighted score	Weighted score	# of equal chains
3	3-3	1.6	48
4	2-2	4.0	48
9	2-2	2.5	12
10	3-1	3.2	12
14	3-1	2.4	6
15	2-1	4.6	6
33	2-1	3.0	12
34	3-0	4.6	12
90	3-0	3.1	

PART 2

# of positions	Unweighted score	Weighted score	# of equal chains
3	3-3	1.6	48
4	3-2	2.2	48
9	2-2	2.5	12
10	3-1	3.2	12
14	3-1	2.4	6
15	3-1	2.0	6
33	2-1	3.0	12
34	3-0	4.6	12
90			

It will be noted that the unweighted mis-match score (in which (9 = 3-3, 8 = 3-2, 7 = 2-2, 6 = 3-1, 5 = 2-1, 4 = 1-1, 3 = 3-0, 2 = 2-0, 1 = 1-0) reduces as the number of

ATAGTC/TATCAG

TACTTC/ATGAAG

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part 2

	AAAACC/TTTTGG	TCTGCT/AGACGA	AAGTAA/TTCATT
	TTGGGG/AACCCC	TCGATA/AGCTAT	CCGTCC/GGCAGG
	TCATAG/AGTATC	ATTCAG/TAAGTC	TGTAAC/ACATTG
5	AGGCTC/TCCGAG	AGAGAT/TCTCTA	ACCGTG/TGGCAC
	GACAAG/CTGTTC	TACTTC/ATGAAG	

33 positions (this chain is obtained at the end of both parts):

10	AACTAG/TTGATC	GTAAGG/CATTCC	TCGCCT/AGCGGA
	TGGAGC/ACCTCG	AAACTA/TTTGAT	TCTCGG/AGAGCC
	TCAAAT/AGTTTA	GTCTCC/CAGAGG	ACCCCC/TGGGGG
	CAGGCC/GTCCGG	ACAGCG/TGTCGC	TTTTCG/AAAAGC
	TATCAC/ATAGTG	CACATC/GTGTAG	AAGTCA/TTCAGT
15	AGATTG/TCTAAG	TGTGTA/ACACAT	GTTCTC/CAAGAG
	TTCCGT/AAGGCA	TAATGC/ATTACG	
	CCCACG/GGGTGC	GGTAAG/CCATTC	
	ATGCCG/TACGGC	AGTTAT/TCAATA	
	TCCGTC/AGGCAG	CAACAG/GTTGTC	
20	CCACGC/GGTGCG	ATCGGC/TAGCCG	
	ACTATG/TGATAC	AATGCT/TTACGA	
	TTAGCA/AATCGT	TTGGAG/AACCTC	

34 positions (this chain is obtained at the end of both parts):

25	AACTCT/TTGAGA	TTATTC/AATAAG	CCAATC/GGTTAG
	TCGAAC/AGCTTG	CACAAG/GTGTTC	ACTTAT/TGAATA
	CAGGGC/GTCCCG	TCCGAT/AGGCTA	AAAGAG/TTTCTC
	TAAAGG/ATTTCC	AGTAGC/TCATCG	TTGATA/AACTAT
30	TGTGCG/ACACGC	CCGTCC/GGCAGC	AAGACC/TTCTGG
	ATGTAG/TACATC	TCACTA/AGTGAT	CAATCC/GTTAGG
	TTCCCC/AAGGGG	GTGACG/CACTGC	TCTCGC/AGAGCG
	AATCTC/TTAGAG	TGAAAT/ACTTTA	AGGGGG/TCCCCC
	TGGCGT/ACCGCA	AGCATG/TCGTAC	TGCCAG/ACGGTC
35	GGCTGC/CCGACG	ACCGTC/TGGCAG	TACTAC/ATGATG

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TTTGAC/AAACTG
ACACCG/TGTGGC
TGAGGC/ACTCCG

5

90 positions (this chain is obtained at the end of part 1) :

	AAAAAA/TTTTTT	TCTGGC/AGACCG	AAACGG/TTTGCC
	CCGGCC/GGCCGG	ACGCAG/TGCGTC	TTTGCC/AAACGG
10	AGGTAG/TCCATC	TGCGTC/ACGCAG	AACCAA/TTGGTT
	TCCATC/AGGTAG	AGTCAT/TCAGTA	CAAAAC/GTTTTG
	ATCTGC/TAGACG	TCAGTA/AGTCAT	AAGGAA/TTCCTT
	TAGACG/ATCTGC	CAGCCG/GTCGGC	CGCCGC/GCGGCG
	ACTGTG/TGACAC	GTCGGC/CAGCCG	AGTGCG/TCACGC
15	TGACAC/ACTGTG	AATTTC/TTAAAG	TCACGC/AGTGCG
	CATTAC/GTAATG	TTAAAG/AATTTC	ATTTTA/TAAAT
	ACCCCA/TGGGGT	CCAACG/GGTTGC	ATCCTA/TAGGAT
	ATGGTA/TACCAT	GGTTGC/CCAACG	AGTATC/TCATAG
	CGAAGC/GCTTCG	CACCAC/GTGGTG	TCATAG/AGTATC
20	ATTACC/TAATGG	AGAATA/TCTTAT	ATGTGG/TACACC
	TAATGG/ATTACC	TCTTAT/AGAATA	TACACC/ATGTGG
	CTCCTC/GAGGAG	ATCAAT/TAGTTA	ATGCAC/TACGTG
	AGTTGA/TCAACT	TAGTTA/ATCAAT	TACGTG/ATGCAC
	AATGCT/TTACGA	ACTTCA/TGAAGT	ACTAAC/TGATTG
25	TTACGA/AATGCT	AGCCCC/TCGGGG	TGATTG/ACTAAC
	AAGCGC/TCGCG	TCGGGG/AGCCCC	CAGTGC/GTCACG
	TTCGCG/AAGCGC	ACCATG/TGGTAC	GTCACG/CAGTGC
	CCCAAG/GGGTTC	TGGTAC/ACCATG	AATAAG/TTATTC
	GGGTTC/CCCAAG	AGGGGA/TCCCCT	TTATTC/AATAAG
30	ACATCC/TGTAGG	CTAATC/GATTAG	AGATAT/TCTATA
	TGTAGG/ACATCC	CGAGAG/GCTCTC	TCTATA/AGATAT
	AACTTG/TTGAAC	GCTCTC/CGAGAG	AAGTCG/TTCAGC
	TTGAAC/AACTTG	ACACGT/TGTGCA	TTCAGC/AAGTCG
	ATAGAC/TATCTG	TGTGCA/ACACGT	AATCGA/TTAGCT
35	TATCTG/ATAGAC	CCTGTC/GGACAG	TTAGCT/AATCGA
	AGACCG/TCTGGC	GGACAG/CCTGTC	AGGCTC/TCCGAG

SP6 primer: 3'-AAGATATCACAGTGGATTAG-5'

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The library components (4 pmol each) were then mixed together and ligated using 100 U T4 DNA ligase (NEB) in 1X ligase buffer at 25°C for 15 minutes. The ligase was then inactivated at 65°C for 20 min.

5 μ l of the ligation reaction (50 μ l) was used as template in a PCR reaction (50 μ l) containing 1X Thermopol buffer (NEB), 0.05 mM dNTPs, 0.4 μ M T7 primer, 0.4 μ M SP6 primer and 0.04 U/ μ l Vent polymerase (NEB). The PCR was hot started (95°C for 3 minutes before addition of polymerase) and cycled 30 times; 95°C, 30 sec; 55°C, 30 sec; 76°C, 30 sec, using a PTC-200 thermo cycler (MJ Research). 10 μ l of the PCR was analysed on a 1.5% agarose gel as shown in Figure 5. The gel picture showed only one intense band corresponding to approximately 240 bp as expected (243 bp). The remaining PCR product was extracted twice with chloroform and precipitated using 71% ethanol and 0.1M NaAc. The DNA was dissolved in water and sequenced. The sequence confirmed that the expected signal chain (1-0-1-0-0) was generated.

EXAMPLE 8 - CONSTRUCTION OF A 5X5 FRAGMENT CHAIN
ENCODING THE BINARY SEQUENCE USING ONE LIGATION CYCLE
FOLLOWED BY ONE PCT CYCLE OR BY TWO LIGATION CYCLES

This experiment demonstrates the use of complementary primer pairs to link fragment chains together as an alternative to the ligation strategy demonstrated in the previous example.

In this experiment 5 fragments chains with 5 positions (fragments or bits) each are ligated separately in ligation cycle 1 as demonstrated earlier (Example 7). The 5 fragment chains are then amplified with 5 different primer pairs (pair 1 is used to amplify chain 1, pair 2 is used to amplify chain 2, etc). The second primer in primer pair 1 is complementary to the first

primer in prime pair 2, the second primer in primer pair 2 is complementary to the first primer in primer pair 3, and so on.

5 A small aliquot is then taken from each of the 5 PCR reactions and a new PCR reactions is performed with primers that are specific to the end of signal chain 1 and 5. The method is illustrated in Figure 6.

10 Materials:

Oligonucleotides are selected which bind to the fragment chain and also serve as primers. Thus for example, for adjacent chains may be bound using for example the
15 following primer pairs:

fragment chain 2 terminal (*with bound primer*):
TTCTATAGTGTCACCTAAATC
AAGATATCACAGTGGATTTAGCCTACCAGTACATCCAACGGCAACT

20 fragment chain 3 terminal (*with bound primer*):
GTCATGTAGGTTGCCGTTGATCCATCCTAATACGACTCACTATAGCA
ATTATGCTGAGTGATATCGT

25 The above exemplified primer regions are complementary and may thus be bound together.

As an alternative to this method, two ligation cycles may be used in which 5 fragment chains (generated by
30 ligation), are ligated together. Thus, several construction cycles to build up long signal chains. After the initial ligation in the first ligation cycle the 5 fragment chains are then amplified with primers containing a *FokI* site. The primers are appropriately
35 selected such that digestion with *FokI* will then make non-palindromic overhangs in the end of each fragment chain in which the overhang generated in fragment chain

1 is able to ligate with the first overhang generated in
fragment chain 2, the second overhang generated in
fragment chain 2 is able to ligate with the first
overhang generated in fragment chain 3, and so on. The 5
5 fragment chains can thereby be ligated together in a
controlled manner to generate a final chain with 25
fragments (bits).

If we want to construct fragment chains with 100 or 500
10 fragments we can repeat this procedure 1 or 2 more
times. The polymerase capacity will, however, be a
limiting factor regarding how many ligation cycles it is
possible to perform. Other strategies will therefore
need to be employed to construct even longer chains.

15
EXAMPLE 9: CLONING OF AN INSERT FROM PHIX174 INTO PUC1
WITH A TRIMMED GENE A

20 This experiment demonstrates the "trimming" strategy for
elimination of unwanted flanking sequences. Another
important aspect of this experiment is that we
demonstrate that it is possible to link a 5' and 3'
overhang together with a single stranded oligonucleotide
alone. It should also be noted that the inserts are
25 cloned into two different IIS sites, thereby eliminating
the problem with insert concatemerisation.

In this method, Gene A from PhiX174 is cloned into a
pUC-19 vector. PhiX174 is prepared by cleavage with
30 BbvI, resulting in 15 fragments flanked by different
non-palindromic 5' 4 bases overhangs, as described in
more detail in Example 1. The two overhangs adjacent to
Gene A is then addressed with "initiation linkers"
containing a BplI site, while the rest of the fragments
35 is allowed to religate. T4 DNA ligase, BplI, a
"propagation linker" containing a BplI site, and two
"termination adaptors" addressed to the first and last

35

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Termination linker 2 (short version):

5'ACC GTC ATT3'

The efficiency of the trimming reaction may be accessed
 as follows. Overhang 6) is addressed with a γ -³²P
 labelled adaptor. The trimming reaction is then allowed
 to start from overhang 1). Aliquots are taken out at
 regularly time intervals and the size distribution of
 the DNA fragments is then analysed on gel.

10

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Claims:

1. A method of synthesizing a double stranded nucleic acid molecule comprising at least the steps of:
- 5 1) generating n double stranded nucleic acid fragments, wherein at least n-2 fragments have single stranded regions at both termini and 2 fragments have single stranded regions at at least one terminus, wherein (n-1) single stranded regions are complementary to (n-1) other
- 10 single stranded regions, thereby producing (n-1) complementary pairs,
- 2) contacting said n double stranded nucleic acid fragments, simultaneously or consecutively, to effect binding of said complementary pairs of single stranded
- 15 regions, and
- 3) optionally ligating said complementary pairs simultaneously or consecutively to produce a nucleic acid molecule consisting of n fragments, wherein said fragment comprises a region representing a
- 20 unit of information corresponding to one or more code elements and said code is alphanumeric.

2. A method of synthesizing a double stranded nucleic acid molecule comprising at least the steps of:
- 25 1) generating n double stranded nucleic acid fragments, wherein at least n-2 fragments have single stranded regions at both termini and 2 fragments have single stranded regions at at least one terminus, wherein (n-1) single stranded regions are complementary to (n-1) other
- 30 single stranded regions, thereby producing (n-1) complementary pairs,
- 2) contacting said n double stranded nucleic acid fragments, simultaneously or consecutively, to effect binding of said complementary pairs of single stranded
- 35 regions, and
- 3) optionally ligating said complementary pairs simultaneously or consecutively to produce a nucleic

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acid molecule consisting of n fragments,
wherein said fragment comprises a region representing a
unit of information corresponding to one or more code
elements and said code is binary.

5

3. A method of synthesizing a double stranded nucleic
acid molecule comprising at least the steps of:

1) generating n double stranded nucleic acid fragments,
10 wherein at least n-2 fragments have single stranded
regions at both termini and 2 fragments have single
stranded regions at at least one terminus, wherein (n-1)
single stranded regions are complementary to (n-1) other
single stranded regions, thereby producing (n-1)
15 complementary pairs,

2) contacting said n double stranded nucleic acid
fragments, simultaneously or consecutively, to effect
binding of said complementary pairs of single stranded
regions, and

20 3) optionally ligating said complementary pairs
simultaneously or consecutively to produce a nucleic
acid molecule consisting of n fragments,
wherein said fragment comprises a region representing a
unit of information corresponding to one or more code
25 elements and each of said one or more code elements has
the formula

$$(X)_a,$$

wherein

30 X is a nucleotide A, T, G, C or a derivative
thereof which allows complementary binding and may be
the same or different at each position, and

a is an integer from 4 to 10,
wherein $(X)_a$ is different for each one or more code
elements.

35

4. A method as claimed in claim 3 wherein said code is
alphanumeric.

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5. A method as claimed in claim 3 wherein said code is binary.

6. A method as claimed in claim 5, wherein said code is binary and the code elements "1" and "0" have the formulae:

"0" = $(X)_a$ and "1" = $(Y)_b$,

wherein

10 $(X)_a$ and $(Y)_b$ are not identical,

X and Y are each a nucleotide A, T, G, C or a derivative thereof which allows complementary binding and may be the same or different at each position, and a and b are integers from 4 to 10.

15

7. A method as claimed in claim 6 wherein in the formulae $(X)_a$ and $(Y)_b$, X and Y are the same at each position.

20 8. A method as claimed in any one of claims 1 to 7 wherein said fragments are each between 8 and 25 bases in length.

25 9. A method as claimed in any one of claims 1 to 8 wherein n is at least 10.

10. A method of synthesizing a double stranded nucleic acid molecule comprising at least the steps of:

30 1) generating fragment chains according to the method defined in any one of claims 1 to 9;

2) optionally generating single stranded regions at the end of said fragment chains, wherein said single stranded regions are complementary to the single stranded regions on said fragment chains thus forming complementary pairs of single stranded regions;

35 3) contacting said fragment chains with one another, simultaneously or consecutively, to effect binding of

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said complementary pairs of single stranded regions.

11. A nucleic acid molecule produced according to a method as defined in any one of claims 1 to 10, or a single stranded nucleic acid molecule thereof.

12. A method of identifying the code elements contained in a nucleic acid molecule prepared according to a method as defined in any one of claims 1 to 10, wherein a probe, carrying a signalling means, specific to one or more code elements, is bound to said nucleic acid molecule and a signal generated by said signalling means is detected, whereby said one or more code elements may be identified.

13. A library of fragments as defined in any one of claims 1 to 12, comprising $(n)_m$ fragments, wherein n is as defined in any one of claims 1 to 12 and corresponds to the length of chain that said library may produce, and m is an integer corresponding to the number of possible code elements or combinations thereof, such that fragments corresponding to all possible code elements for each position in the final chain are provided.

14. A kit for synthesizing a double stranded nucleic acid molecule comprising a library as defined in claim 13 and a ligase.

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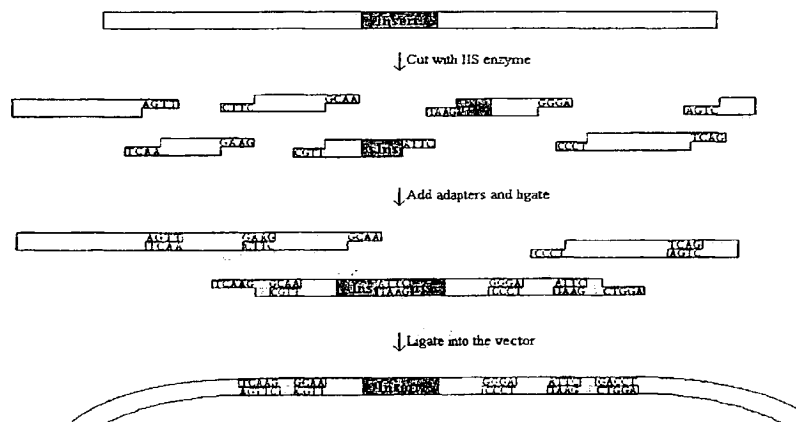
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- (72) Inventor; and
(75) Inventor/Applicant (for US only): LEXOW, Preben [NO/NO]; Bloksbergveien 16, N-3132 Husøysund (NO).
- (74) Agents: JONES, Elizabeth, Louise et al.; Frank B. Dehn & Co., 179 queen Victoria Street, London EC4V 4EL (GB).
- (81) Designated States (national): AE, AG, AL, AM, AT, AT (utility model), AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, CZ (utility model), DE, DE (utility model), DK, DK (utility model), DM, DZ, EE, EE (utility model), ES, FI, FI (utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KR (utility model), KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (utility model), SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.
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[Continued on next page]

(54) Title: METHODS OF CLONING AND PRODUCING FRAGMENT CHAINS WITH READABLE INFORMATION CONTENT



(57) Abstract: The present invention provides a method of attaching a fragment of a first nucleic acid molecule to a second nucleic acid molecule using adapters to mediate the binding, particularly in methods of cloning, methods of producing fragment chains with a readily readable information content, particularly comprising fragments corresponding to code, such as alphanumeric code, the nucleic acid molecules thus produced and kits for performing such methods.

WO 01/00816 A1

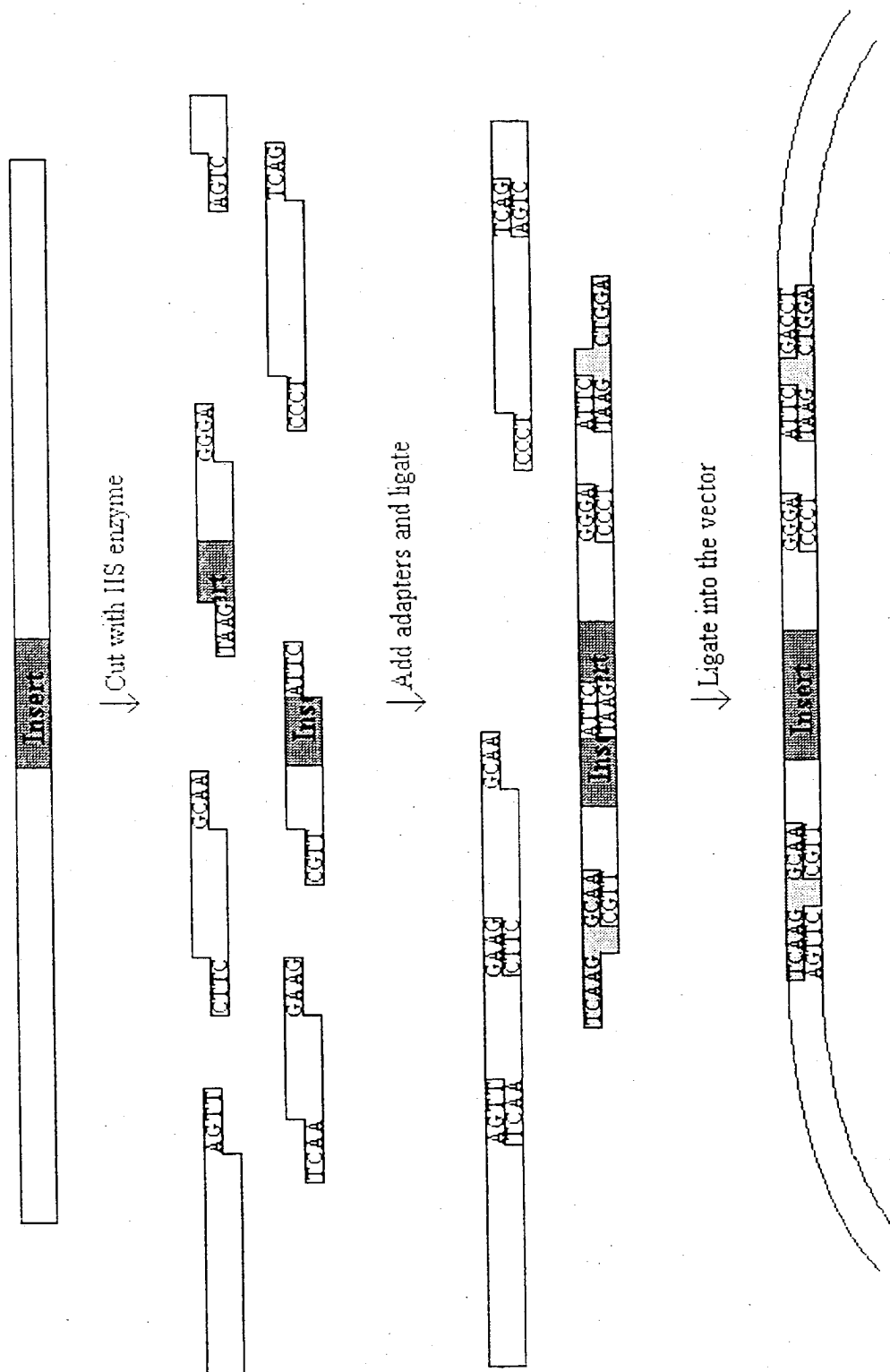


FIG. 1

2 / 6

	«0» starting fragments:	«1» starting fragments:
Position 1	GGGG GGGGAAA CCCCCCCCC	AAAAAAAAAAA TTTTTTTTT
Position 2	GGGG GGGGAAC TTTCCCCCCCC	AAAAAAAAAAC TTTTTTTTTTTT
	:	
	:	
	:	
	:	
Position 7	GGGG GGGGCCG GCGCCCCCCCC	AAAAAAACCG GCGTTTTTTTT
Position 8	GGGG GGGG GGCCCCCCCCC	AAAAAAA GGCTTTTTTTTT

FIG. 2

	Fragment 0	Fragment 1
Position 1.1	GGGG GGGGAAA CCCCCCCCC	AAAAAAAAAAA TTTTTTTTT
Position 1.2	AAAGGGG GGGGAAA CCCCCCCCC	AAAAAAAAAAAAA TTTTTTTTT
Position 1.3	AACGGGG GGGGAAA CCCCCCCCC	AACAAAAAAAAAA TTTTTTTTT
	:	
	:	
	:	
Position 8.1	GGGG GGGG GCCCCCCCCCCTTT	AAAAAAA GCTTTTTTTTTTT
Position 8.2	GGGG GGGG GCCCCCCCCCCTTG	AAAAAAA GCTTTTTTTTTTTG
Position 8.3	GGGG GGGG GCCCCCCCCCCTTC	AAAAAAA GCTTTTTTTTTTTC
	:	
	:	

FIG. 3

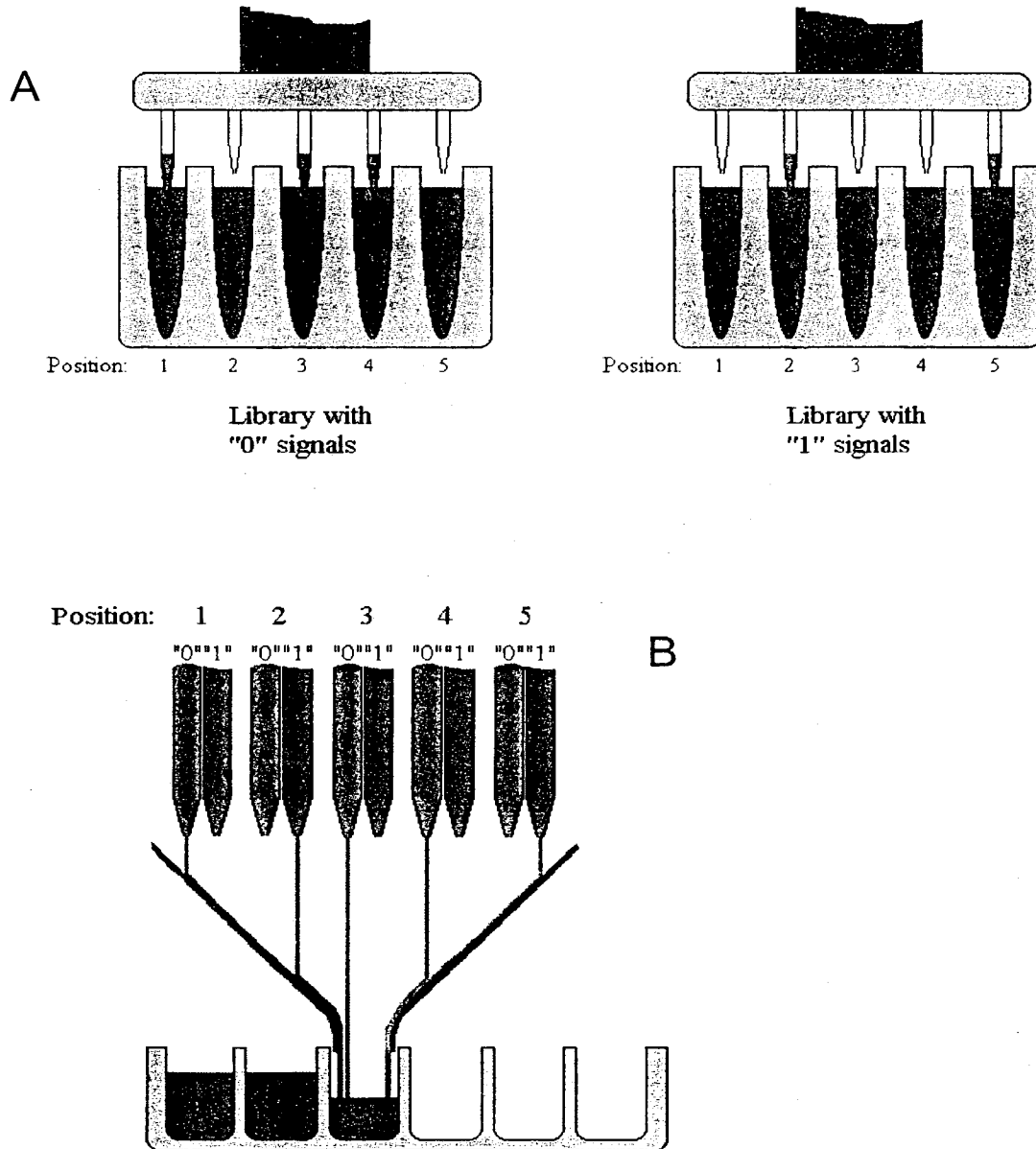


FIG. 4

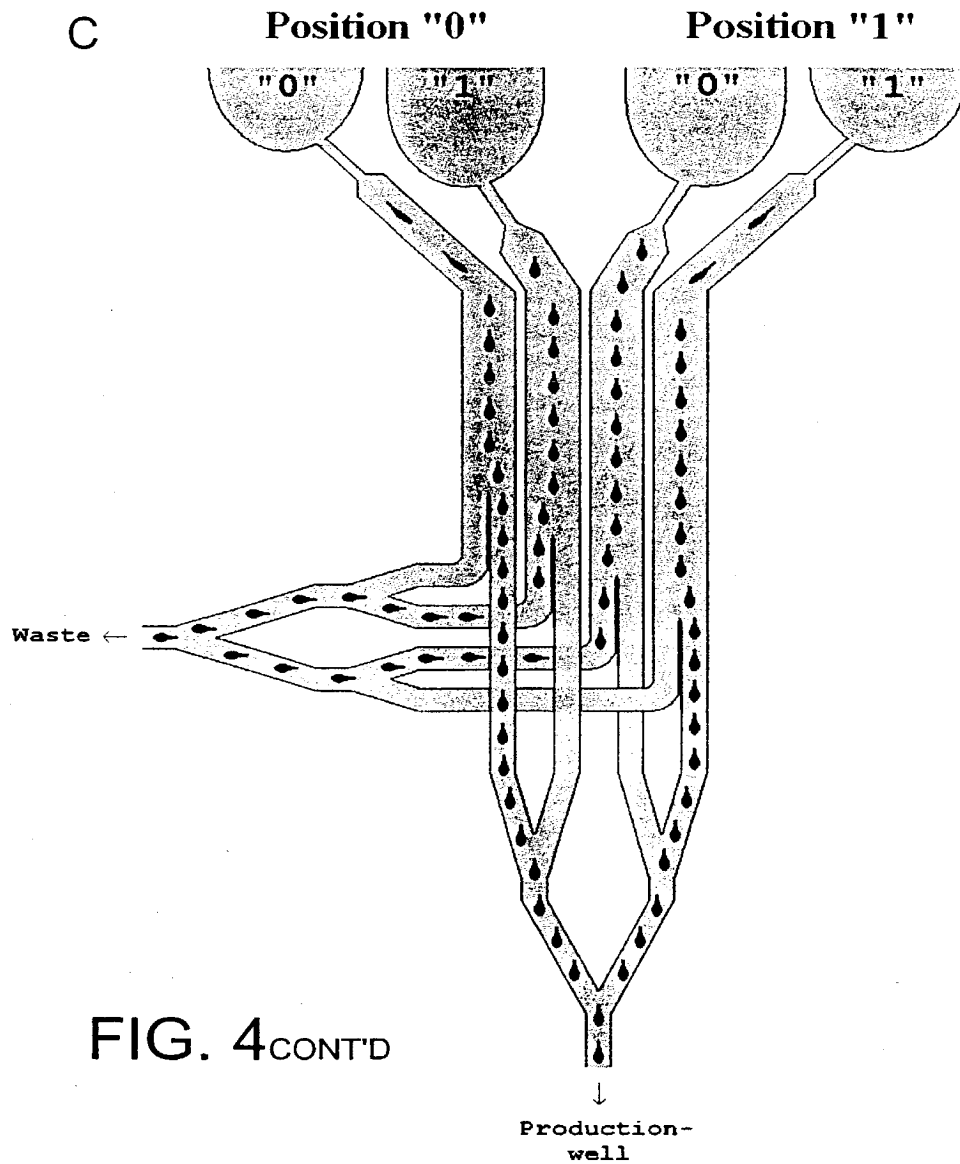


FIG. 4CONT'D

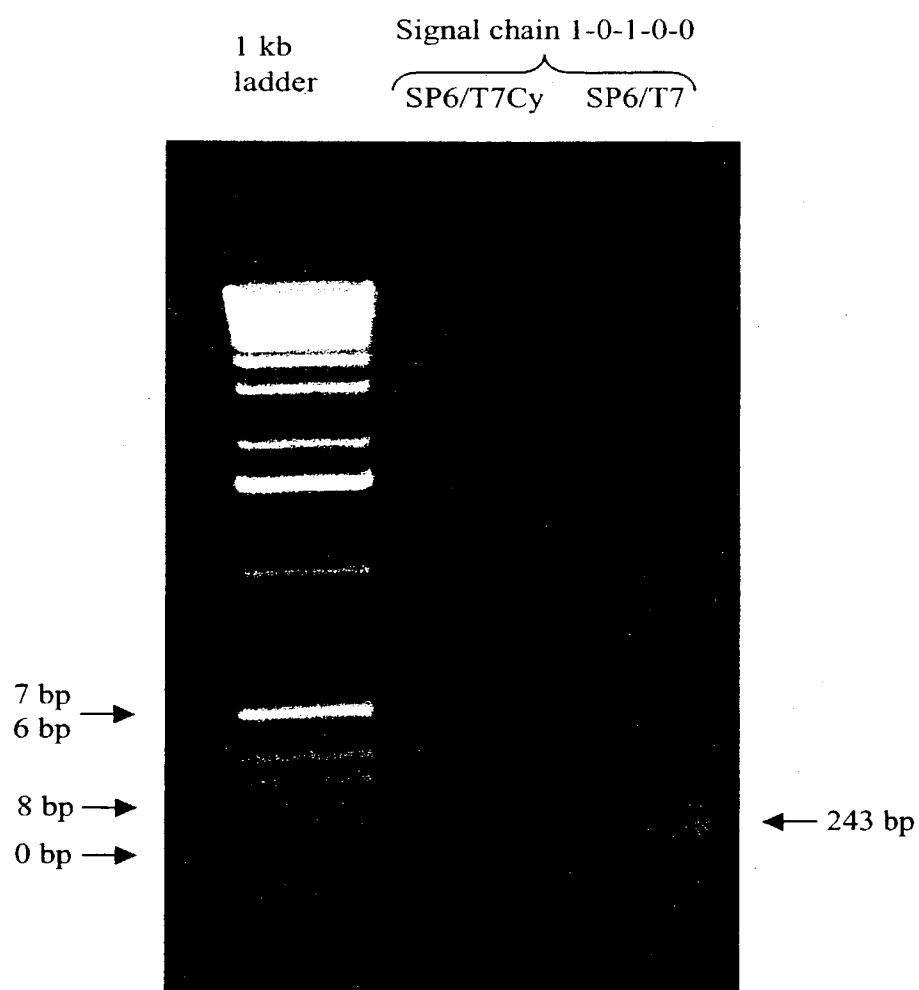


FIG. 5

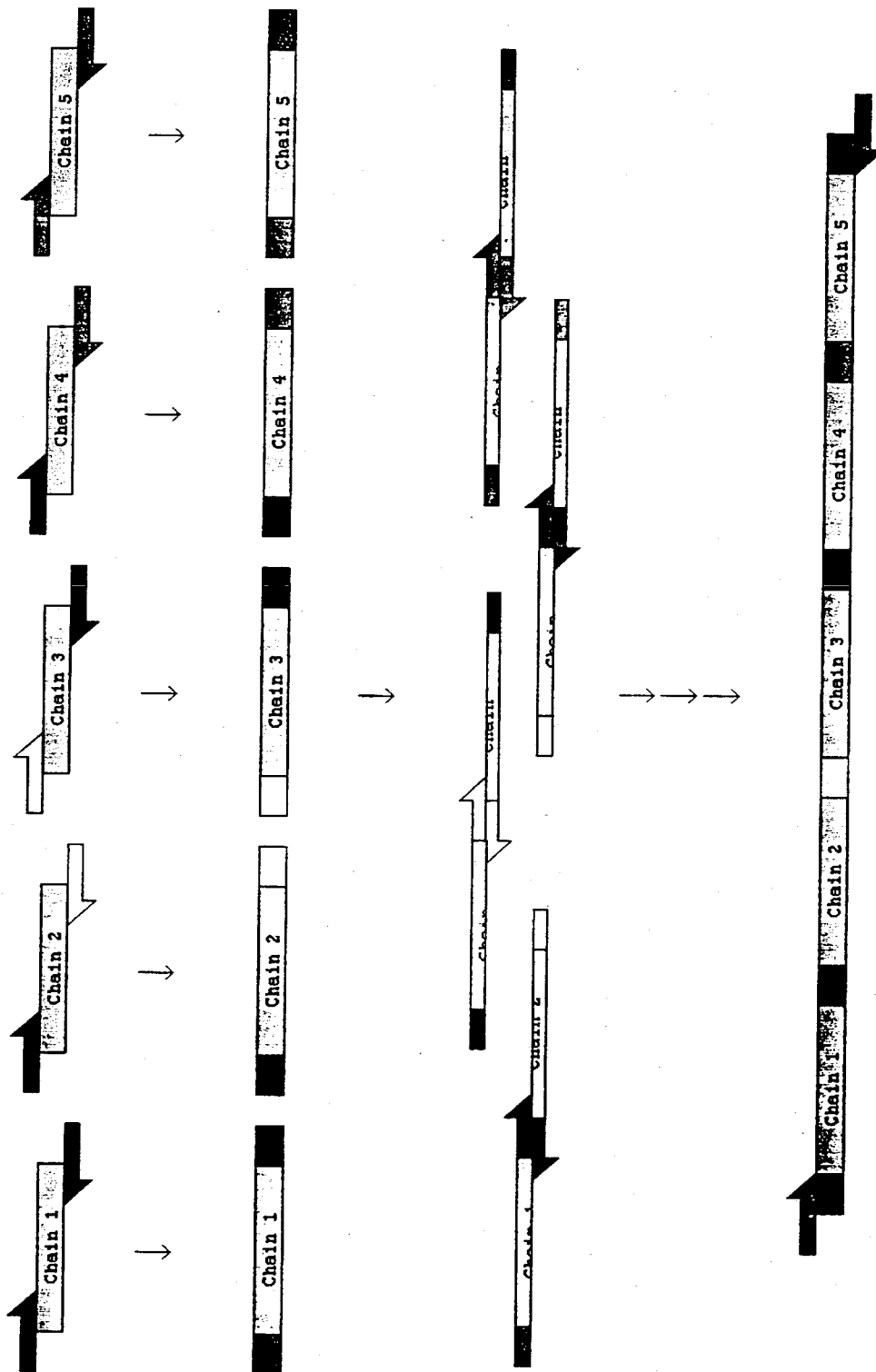


FIG. 6

DECLARATION AND POWER OF ATTORNEY FOR UTILITY OR DESIGN PATENT APPLICATION (37 CFR 1.63) <input type="checkbox"/> Declaration Submitted with Initial Filing <input checked="" type="checkbox"/> Declaration Submitted after Initial Filing		Attorney Docket No.	1181-256
		First Named Inventor	Preben LEXOW
		COMPLETE IF KNOWN	
		Application Number	
		Filing Date	
		Group Art Unit	
		Examiner Name	

As a below named inventor, I hereby declare that:

My residence, mailing address, and citizenship are as stated below next to name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: **METHODS OF CLONING AND PRODUCING FRAGMENT CHAINS WITH READABLE INFORMATION CONTENT** the specification of which is was filed on June 27, 2000 as PCT International Application Number PCT/GB00/02512.

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56, including for continuation-in-part applications, material information which became available between the filing date of the prior application and the national or PCT international filing date of the continuation-in-part application.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Numbers	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached?	
				YES	NO
19991325	NO	06/28/1999	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
20003190	NO	06/20/2000	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
20003191	NO	06/20/2000	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

I hereby claim the benefit under 35 U.S.C. 119(e) of any United States provisional application(s) listed below.

Application Number(s)	Filing Date (MM/DD/YYYY)

I or we hereby appoint the registered practitioner(s) associated with Customer No. 6449 to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith. Direct all correspondence to Customer Number 6449.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

NAME OF SOLE OR FIRST INVENTOR:		<input type="checkbox"/> A petition has been filed for this unsigned inventor	
Given Name (first and middle [if any]) <u>Preben</u>		Family Name or Surname <u>LEXOW</u>	
Inventor's Signature <u>Helen LEXOW</u>		Date <u>21 September 2002</u>	
Residence: City	<u>Husoyund</u>	State	Country <u>Norway</u> Citizenship <u>Norway</u>
Mailing Address	<u>Bloksbergveien 16</u>		
Mailing Address			
City	<u>Husoyund</u>	Postal Code	<u>N-3132</u> Country <u>Norway</u>

10/0192585

#

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SEQUENCE LISTING

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<220>

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<221> misc_feature
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 <223> N is any nucleotide.

<400> 2
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11

<210> 3
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<220>
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<400> 3
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23

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24

<210> 5
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<400> 5
 ctctgcgcct ccagtgcagc ggag

24

<210> 6
 <211> 24
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<220>
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<400> 6
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<210> 7
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<220>
 <223> BbvI overhang 7a

<400> 7
 caacgcgcct ccagtgcagc ggag

24

<210> 8
 <211> 24
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<220>
 <223> BbvI overhang 9b

<400> 8
 ggtagcgcct ccagtgcagc ggag

24

<210> 9
 <211> 25
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Cloning site 1a

<400> 9
 aagagctccg ctgcactgga ggcgc

25

<210> 10
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 <213> Artificial Sequence

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<400> 10
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25

<210> 11
 <211> 35
 <212> DNA
 <213> Artificial Sequence

<220>
<223> Consensus binding motifs of the initiation linkers

<220>
<221> misc_feature
<222> (19)..(24)
<223> N is any nucleotide.

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gcagcgacca tgagtccanc tcnngtggat gacgc

35

<210> 12
<211> 37
<212> DNA
<213> Artificial Sequence

<220>
<223> Initiation linker

<220>
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2 to 37 is not palindromic.

<400> 12
gcagcgacca tgagtccanc tcnngtggat gnnnnnn

37

<210> 13
<211> 38
<212> DNA
<213> Artificial Sequence

<220>
<223> Initiation linker

<220>
<221> misc_feature
<222> (19)..(38)
<223> N is any nucleotide with the proviso that the DNA sequence from 3
3 to 38 is not palindromic.

<400> 13
gcagcgacca tgagtccanc tcnngtggat gnnnnnnnn

38

<210> 14
<211> 39
<212> DNA
<213> Artificial Sequence

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<220>
<223> Initiation linker

<220>
<221> misc_feature
<222> (19)..(39)
<223> N is any nucleotide with the proviso that the DNA sequence from 3
      4 to 39 is not palindromic.

<400> 14
gcagcgacca tgagtcanc tcnngtggat gnnnnnnnnn 39

<210> 15
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> Initiation linker

<220>
<221> misc_feature
<222> (19)..(40)
<223> N is any nucleotide with the proviso that the DNA sequence from 3
      5 to 40 is not palindromic.

<400> 15
gcagcgacca tgagtcanc tcnngtggat gnnnnnnnnnn 40

<210> 16
<211> 41
<212> DNA
<213> Artificial Sequence

<220>
<223> Initiation linker

<220>
<221> misc_feature
<222> (19)..(41)
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gcagcgacca tgagtcanc tcnngtggat gacgcnnnnn n 41

<210> 17
<211> 42

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<212> DNA
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 <223> Initiation linker
 <220>
 <221> misc_feature
 <222> (19)..(42)
 <223> N is any nucleotide with the proviso that the DNA sequence from 3
 7 to 42 is not palindromic.

<400> 17
 gcagcgacca tgagtccanc tcnngtggat gacgcnnnnn nn 42

<210> 18
 <211> 43
 <212> DNA
 <213> Artificial Sequence
 <220>
 <223> Initiation linker
 <220>
 <221> misc_feature
 <222> (19)..(43)
 <223> N is any nucleotide with the proviso that the DNA sequence from 3
 8 to 43 is not palindromic.

<400> 18
 gcagcgacca tgagtccanc tcnngtggat gacgcnnnnn nnn 43

<210> 19
 <211> 44
 <212> DNA
 <213> Artificial Sequence
 <220>
 <223> Initiation linker
 <220>
 <221> misc_feature
 <222> (19)..(44)
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 9 to 44 is not palindromic.

<400> 19
 gcagcgacca tgagtccanc tcnngtggat gacgcnnnnn nnnn 44

<210> 20
 <211> 45
 <212> DNA
 <213> Artificial Sequence

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 <223> Initiation linker

<220>
 <221> misc_feature
 <222> (19)..(45)
 <223> N is any nucleotide with the proviso that the DNA sequence from 4
 0 to 45 is not palindromic.

<400> 20
 gcagcgacca tgagtccanc tcnngtggat gacgcnnnnn nnnnn 45

<210> 21
 <211> 46
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Initiation linker

<220>
 <221> misc_feature
 <222> (19)..(46)
 <223> N is any nucleotide with the proviso that the DNA sequence from 4
 1 to 46 is not palindromic.

<400> 21
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<210> 22
 <211> 50
 <212> DNA
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<400> 22
 taatacgact cactatacca caagtttgta caaaaaagca ggctctattc 50

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<400> 23
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<400> 24
 ttcctatgca gtggaccact ttgtacaaga aagctggggt gcagt 45

<210> 25
 <211> 45
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<220>
 <223> Synthetic oligonucleotide

<400> 25
 gcaactactg caaccagct ttcttgtaca aagtgtcca ctgca 45

<210> 26
 <211> 45
 <212> DNA
 <213> Artificial Sequence

<220>
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<400> 26
 agttgcttga cgccacaagt ttgtacaaaa aagcaggctt tgacg 45

<210> 27
 <211> 45
 <212> DNA
 <213> Artificial Sequence

<220>
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<400> 27
 cgacatcgtc aaagcctgct tttttgtaca aacttgtggc gtcaa 45

<210> 28
 <211> 45
 <212> DNA
 <213> Artificial Sequence

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 <223> Synthetic oligonucleotide

<400> 28
 atgtcgaagg gcggaccact ttgtacaaga aagctgggta agggc

45

<210> 29
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<400> 29
 gacagggccc ttaccagct ttcttgata aagtgggccg ccctt

45

<210> 30
 <211> 58
 <212> DNA
 <213> Artificial Sequence

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<400> 30
 cctgtcatgt ggaccacttt gtacaagaaa gctgggtttc tatagtgtca cctaaatc

58

<210> 31
 <211> 52
 <212> DNA
 <213> Artificial Sequence

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<400> 31
 gatttaggtg acactataga aaccagctt tcttgataa agtgggtccac at

52

<210> 32
 <211> 20
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<400> 32
taatacgact cactatacca 20

<210> 33
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<220>
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<400> 33
taatacgact cactata 17

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<400> 34
aagatatcac agtggattta g 21

<210> 35
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<223> Fragment chain 2 terminal

<400> 35
ttctatagtg tcacctaaat c 21

<210> 36
<211> 46
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<213> Artificial Sequence

<220>
<223> Primer

<400> 36
tcaacggcaa cctacatgac catccgattt aggtgacact atagaa 46

<210> 37
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tgctatagtg agtcgtatta		20
<210>	39	
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cgactgagag catctcgacc gaat		24
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gcgttactga gcgtagctct g 21

<210> 42
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<400> 42
 ctctcagagc tacgctcagt aacgc 25

<210> 43
 <211> 24
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 <223> Propagation linker (s)

<220>
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 <222> (20)..(24)
 <223> N is any nucleotide.

<400> 43
 tgctgcagga gcgaatctcn nnnn 24

<210> 44
 <211> 19
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<400> 44
 gagattcgct cctgcagca 19

<210> 45
 <211> 24
 <212> DNA
 <213> Artificial Sequence

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 <223> Labeling linker 2 (s)

<400> 45
 ctcttgctat agtgagtcgt atta 24

<210> 46
 <211> 20
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Labeling linker 2 (as)

<400> 46
 taatacgact cactatagca 20

<210> 47
 <211> 30
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 <213> Artificial Sequence

<220>
 <223> Termination linker 1 (s)

<400> 47
 aagagctcag gtcattgacg tagctatgaa 30

<210> 48
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 <223> Termination linker 1/2 (as)

<400> 48
 agctacgtca atgacctgag 20

<210> 49
 <211> 10
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 <213> Artificial Sequence

<220>
 <223> Termination linker 1 (short version)

<400> 49
 aagagatgaa 10

<210> 50
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 <213> Artificial Sequence

<210> 55
 <211> 11
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> 0 starting fragment, postion 7

<400> 55
 gggggggggcc g

11

<210> 56
 <211> 12
 <212> DNA
 <213> Artificial Sequence

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 <223> 0 starting fragment, postion 7

<400> 56
 ccccccccg cg

12

<210> 57
 <211> 10
 <212> DNA
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<220>
 <223> 1 starting fragment, postion 7

<400> 57
 aaaaaaacg

10

<210> 58
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<400> 58
 ttttttttgc g

11

<210> 59
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 <223> 0 starting fragment, postion 8

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cccccccccc gg 12

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tttttttttcg g 11

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aacaaaaaaa aaa 13

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13

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catccacnng agntggactc atggtcgctg c

31

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<223> N is any nucleotide.
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ncatccacnn gagntggact catggtcgct gc
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32

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<221> misc_feature
<222> (1)..(15)
<223> N is any nucleotide.
```

<400> 71
nncatccacn ngagntggac tcatggtcgc tgc

33

<210>	72
<211>	34
<212>	DNA
<213>	Artificial Sequence

```
<220>
<223> Initiation linker
```

```
<220>
<221> misc_feature
<222> (1)..(16)
<223> N is any nucleotide.
```

```
<400> 72
nnncatccac nngagntgga ctcatggtcg ctgc
```

<210>	73
<211>	35
<212>	DNA
<213>	Artificial Sequence

```
<220>
<223> Initiation linker
```

```
<220>
<221> misc_feature
<222> (12)..(17)
<223> N is any nucleotide.
```

<400> 73
gcgtcatcca cnnagantgg actcatggtc gctgc

<210>	74
<211>	36
<212>	DNA
<213>	Artificial Sequence

```
<220>  
<223> Initiation linker
```

```
<220>
<221> misc_feature
<222> (1)..(18)
<223> N is any nucleotide.
```

```
<400> 74
nqcgtcatcc acnngagntg gactcatggt cgctgc
```

<210> 75
 <211> 37
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Initiation linker

<220>
 <221> misc_feature
 <222> (1)..(19)
 <223> N is any nucleotide.

<400> 75
 nngcgatcatc cacnngagnt ggactcatgg tcgctgc

37

<210> 76
 <211> 38
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Initiation linker

<220>
 <221> misc_feature
 <222> (1)..(20)
 <223> N is any nucleotide.

<400> 76
 nnnngcgatcat ccacnngagn tggactcatg gtcgctgc

38

<210> 77
 <211> 39
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Initiation linker

<220>
 <221> misc_feature
 <222> (1)..(21)
 <223> N is any nucleotide.

<400> 77
 nnnngcggtca tccacnngag ntggactcat ggtecgctgc

39

<210> 78
 <211> 40
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Initiation linker

<220>
 <221> misc_feature
 <222> (1)..(22)
 <223> N is any nucleotide.

<400> 78
 nnnnngcgtc atccacnnga gntggactca tggtcgctgc

40

<210> 79
 <211> 10
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Propagation linker HgaI

<220>
 <221> misc_feature
 <222> (1)..(5)
 <223> N is any nucleotide.

<400> 79
 nnnnngcgtc

10

<210> 80
 <211> 32
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Gene A from PHIX174

<400> 80
 gctggaggcc tccactatga aatcgcgtag ag

32

<210> 81
 <211> 28
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Gene A from PHIX174

<400> 81
ctggcggaat atgagaaaat tcgaccta

28

<210> 82
<211> 13
<212> DNA
<213> Artificial Sequence

<220>
<223> Recognition motif of the N-terminal part of the hsdS subunit of S
tyR 1241

<220>
<221> misc_feature
<222> (4)..(9)
<223> N is any nucleotide.

<400> 82
gaannnnnnr tcg

13

<210> 83
<211> 14
<212> DNA
<213> Artificial Sequence

<220>
<223> Recognition motif of the C-terminal part of the hsdS subunit of S
tyR 1241

<220>
<221> misc_feature
<222> (4)..(10)
<223> N is any nucleotide.

<400> 83
tcannnnnnn rttc

14

<210> 84
<211> 13
<212> DNA
<213> Artificial Sequence

<220>
<223> Recognition motif of a new enzyme made by merging the N- and C-te
rminal parts of the hsdS subunit of StyR 1241

<220>
<221> misc_feature
<222> (4)..(9)

<223> N is any nucleotide.

<400> 84

gaannnnnnr ttc

13

<210> 85

<211> 40

<212> DNA

<213> Artificial Sequence

<220>

<223> Ligated initiation linker

<220>

<221> misc_feature

<222> (1)..(22)

<223> N is any nucleotide with the proviso that the sequence from 1 to 6 is complementary to the sequence from 40 to 35 of SEQ ID NO: 15

<400> 85

nnnnnnnnnc atccacnnga gntggactca tggtcgctgc

40

<210> 86

<211> 47

<212> DNA

<213> Artificial Sequence

<220>

<223> An example of sequences that generate 5'-4 base overhangs by BbsI and Esp3I

<220>

<221> misc_feature

<222> (1)..(47)

<223> N is any nucleotide.

<400> 86

nnnnnnnnnga gcngagacgn nnnnnngaaga cngagacnnn nnnnnnn

47

<210> 87

<211> 47

<212> DNA

<213> Artificial Sequence

<220>

<223> An example of sequences that generate 5'-4 base overhangs by BbsI and Esp3I

```
<400> 87
nnnnnnnnnnn gctenngtct tennnnnnncg tctengctcn nnnnnnn
```

<210>	88
<211>	29
<212>	DNA
<213>	Artificial Sequence

<220>
<223> An example of 5' -4 base overhangs generated by BbsI and Esp3I cleavage

```
<220>
<221> misc_feature
<222> (5)..(25)
<223> N is any nucleotide.
```

<400> 88
gagcngagac gnnnnnnngaa gacnngagc

<210>	89
<211>	25
<212>	DNA
<213>	Artificial Sequence

<220>
<223> An example of 5' -4 base overhangs generated by BbsI and Esp3I cleavage

```
<220>
<221> misc_feature
<222> (5)..(25)
<223> N is any nucleotide.
```

```
<400> 89
getcnngtct tcnnnnnnneg tctcn
```

<210>	90
<211>	22
<212>	DNA
<213>	Artificial Sequence

<220>
<223> An example of ligation products between 5' -4 base overhangs gene

rated by BbsI and Esp3I cleavage

<220>
 <221> misc_feature
 <222> (1)..(22)
 <223> N is any nucleotide.

<400> 90
 nnnnnnnnga gcnnnnnnnn nn 22

<210> 91
 <211> 22
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> An example of ligation products between 5' -4 base overhangs gene
 rated by BbsI and Esp3I cleavage

<220>
 <221> misc_feature
 <222> (1)..(22)
 <223> N is any nucleotide.

<400> 91
 nnnnnnnnnn gctcnnnnnn nn 22

<210> 92
 <211> 51
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> An example of sequences that generate two 3' 3 base overhangs by
 BsaXI

<220>
 <221> misc_feature
 <222> (1)..(51)
 <223> N is any nucleotide.

<400> 92
 nnnnnnnnga gnnnnnnnnn acnnnnnctc cnnnnnnnga gnnnnnnnnn n 51

<210> 93
 <211> 51
 <212> DNA
 <213> Artificial Sequence

$\langle 220 \rangle$

<223> An example of sequences that generate two 3' 3 base overhangs by BsaXI

 $\langle 220 \rangle$

<221> misc feature

$$\langle 222 \rangle \quad (1) \cdot \cdot (51)$$

<223> N is any nucleotide.

<400> 93

nnnnnnnnnnn ctcnnnnnnnn ggagnnnnnnng tnnnnnnnnnn ctcnnnnnnnn n

51

<210> 94

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> An example of 3' 3 base overhangs generated by BsaXI cleavage

 $\langle 220 \rangle$

```
<221> misc feature
```

$$\langle 222 \rangle \quad (1) \cdot \bar{\cdot} (27)$$

<223> N is any nucleotide.

<400> 94

nnnnnnnnna cnnnnnctcc nnnnnnnngag

30

<210> 95

<211> 30

<212> DNA

<213> Artificial Sequence

 $\langle 220 \rangle$

<223> An example of 3' 3 base overhangs generated by BsaXI cleavage

 $\langle 220 \rangle$

<221> misc feature

$$\langle 222 \rangle \quad (1) \cdot \bar{\cdot} (27)$$

<223> N is any nucleotide.

<400> 95

nnnnnnnngga gnnnnngttn nnnnnnnnctc

30

<210> 96

<211> 44

<212> DNA

<213> Artificial Sequence

SECRET

```
<400> 96
nnnnnnnnnn nnnnnnnnnn nnnngagtcn nnnnnnnnnn nnnn
```

44

<210>	97
<211>	26
<212>	DNA
<213>	Artificial Sequence

```
<220>
<223> An example of 3' 3 base overhangs generated by MlyI cleavage
.
<220>
<221> misc_feature
<222> (1)..(26)
<223> N is any nucleotide.
```

```
<400> 97
nnnnnnnnnnnn nnnnnngagt cnnnnn
```

26

<210>	98
<211>	30
<212>	DNA
<213>	Artificial Sequence

<220>
<223> Gene A from PHIX174

```
<400> 98
ctacgcgatt tcatagtgga ggcctccagc
```

30

<210>	99
<211>	28
<212>	DNA
<213>	Artificial Sequence

<220>
<223> Gene A from PHIX174

<400> 99
ggtcgaattt tctcattttc cgccagca

28

<210> 100
 <211> 10
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> 1 starting fragment, position 1

<400> 100
 aaaaaaaaaa

10

<210> 101
 <211> 11
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> 1 starting fragment, position 2

<400> 101
 tttttttttt t

11

<210> 102
 <211> 13
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Fragment 1, position 1.2

<400> 102
 aaaaaaaaaa aaa

13

<210> 103
 <211> 14
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Fragment 0, position 1.3

<400> 103
 aacggggggg gaaa

14

<210> 104
 <211> 14
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Fragment 0, position 8.3

<400> 104
cttccccccc cccg

14

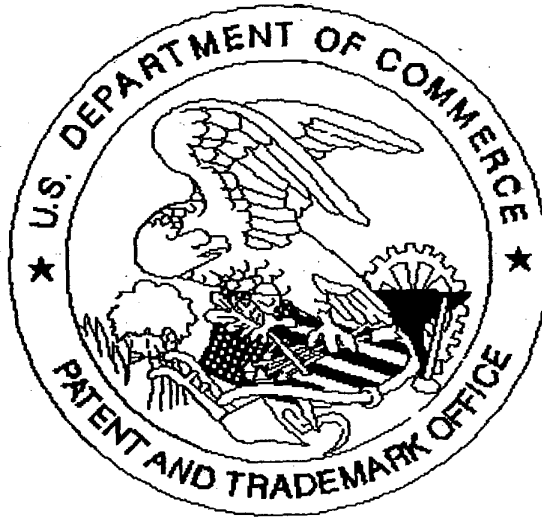
<210> 105
<211> 13
<212> DNA
<213> Artificial Sequence

<220>
<223> Fragment 1, position 8.3

<400> 105
cttttttttt tcg

13

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